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Table of Contents

Foreward

Progress is summarized on our project to investigate the diagnostic and therapeutic potential for Bin1 in prostate cancer. We have successfully generated 9 new monoclonal antibodies capable of recognizing all Bin1 splice isoforms that have been identified in cells. These antibodies are currently being tested and optimized for staining of fixed tissues. In Aim 2, we proposed to perform immunohistochemical studies. To date, we have analyzed 30 cases of frozen primary prostate cancer and found that 29/30 retained Bin1 expression in epithelial cells. Additional cases including metastatic cancers with follow-up information are pending completion of antibody testing. We believe this line of work will prove informative, because Northern analysis of primary and metastatic cancers confirms frequent expression in primaries but indicates universal losses in metastatic tumors. To date, gene status has been examined in 15 primary tumors. Using a heterozygous marker within the Bin1 gene we have documented loss of heterozygosity (LOH) in 6/15 of these tumors (40% LOH rate). However, the remaining alleles in tumors exhibiting LOH did not show alteration or perhaps only polymorphism rather than mutation. Metastatic variants of these tumors were not available to learn whether mutation had occured later in progression. This possibility is viable insofar as we have observed expression of normal Bin1 message in androgen-dependent LNCaP cells but misspliced messages that encode inactive polypeptides in androgen-independent PC3 and DU145 cells. Further analysis of Bin1 in metastatic tumors is planned as is an examination of promoter methylation status. Functional investigations indicate that LNCaP and PC3 are relatively susceptible to growth inhibition by Bin1 whereas DU145 is relatively insensitive. Adenoviral vectors for Bin1 are nonspecifically toxic in our hands. Therefore, as an alternate and more satisfying physiological approach, we hope to cross Bin1 "knockout" mice that have been developed to prostate tumor-prone transgenic animals, as a way to accurately gauge the significance of Bin1 loss in prostate cancer development and its mechanism of action in apoptosis.

Introduction

The genetic causes of prostate cancer remain largely unknown. One of the most common chromosomal abnormalities seen in tumors which have acquired invasive and metastatic potential is gain of chromosome 8p, where c-Myc is located (Bova and Isaacs 1996). Gains of 8q of are well-correlated with disease progression insofar as they are found in 85% of lymph node metastases and 89% of recurrent hormone refractive tumors (Cher *et al.* 1996; Van Den Berg *et al.* 1995; Visakorpi *et al.* 1995). c-Myc amplification or overexpression is found in many prostate tumors and is a likely progression marker (Buttyan *et al.* 1987; Fleming *et al.* 1986; Jenkins *et al.* 1997). Oncogenic activation of c-Myc by gene amplification delivers a powerful signal that is sufficient to drive cell cycle progression and malignant growth in many types of cells, including prostate cells (Thompson *et al.* 1989). However, in premalignant cells, c-Myc can also activate apoptosis such that its oncogenic activation is balanced by apoptotic penalty (Prendergast 1999). Therefore, malignant cells may escape this penalty by inactivating tumor suppressor functions. p53 is an important player but is likely irrelevant to this process in prostate cells because inactivation of p53 does not compromise c-Myc-mediated apoptosis in epithelial cells (Sakamuro *et al.* 1995; Trudel *et al.* 1997). Thus, loss or inactivation of molecules other than p53 should be considered.

c-Myc lies an intersection of two signaling networks which target its C-terminal DNA binding domain and its N-terminal transcriptional transactivation domain (Sakamuro and Prendergast 1999). Recent advances have led to the identification of a set of novel N-terminal-interacting proteins which

constitute a second Myc network (Sakamuro and Prendergast 1999). One of these proteins, Bin1, is a ubiquitous adaptor protein which has features of a tumor suppressor (Elliott *et al.* 1999; Sakamuro *et al.* 1996) that has been linked to cell death and differentiation decisions (Sakamuro and Prendergast 1999). We hypothesized that Bin1 might be inactivated in prostate cancer because the human Bin1 gene maps to chromosome 2q14 (Negorev *et al.* 1996), within a region of chromosome 2q that is frequently deleted in metastatic prostate tumors (Brothman 1997; Cher *et al.* 1996) but where no tumor suppressor gene has been identified to date. Since c-Myc is frequently amplified in prostate carcinomas and Bin1 can suppress malignant transformation by c-Myc (Sakamuro *et al.* 1996), loss of Bin1 activity would eliminate a mechanism that can limit full oncogenic activation of c-Myc.

Progress on aims to examine the significance of Bin1 in prostate cancer follow.

Aim 1. Develop monoclonal antibodies that can detect Bin1 in fixed tissues.
Tasks 1, 2, and 3 were completed. Task 4 is in progress.

Aim 2. Perform an immunohistological analysis of Bin1 in staged primary tumors and metastases.
Task 1 to gather, catalog, and grade 30 frozen tumors was completed. Task 2 to accumulate fixed tumor sections of different grades is pending completion of Aim 1, Task 4. Tasks 3 and 4 to perform immunohistochemistry are partlially completed pending completion of Aim 1, Task 4.

Aim 3. Identify mutations in the Bin1 gene in androgen-independent tumor cells.
Tasks 1-4 to analyze the Bin1 gene in primary prostate tumors is partly completed. Task 5 to assay genetic mutations is deferred pending discovery of such mutations.

Aim 4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.
Tasks were changed following the determination that the adenoviral vectors in use were exhibiting nonspecific toxicity on prostate cancer cell lines in our hands. Colony formation assays using plasmid vectors (alternate strategy for Tasks 1 and 2) were completed. For an alternate to Task 3, which is to examine the in vivo role of Bin1, we plan to exploit a "knockout" mouse model that has been developed. Task 4 to examine the mechanism of apoptosis by Bin1 is in progress using cells isolated from "knockout" mice.

Body

Aim 1. Develop monoclonal antibodies that can detect Bin1 in fixed tissues.

Tasks 1,2,3 to develop a new set of monoclonal antibodies are completed. We have obtained nine (9) new antibodies that recognize 4 different epitopes in the N-terminal BAR domain of Bin1. This domain is found in all the various splice isoforms of Bin1 that we have been characterized in cells. The antibodies have been shown to be specific and effective for immunoprecipitation (5/9 antibodies), immunofluorescence (5/9 antibodies), and Western analyses (9/9 antibodies). Two of the antibodies obtained are quite avid, based on their ability to immunoprecipitate Bin1 in extraction buffers containing 0.1% SDS (i.e. RIPA conditions), and most give excellent signal-to-noise on Western analysis of Bin1 directly from cell extracts. We are currently carrying out Task 4, which is to test the ability of antibodies to effectively stain fixed prostate tissue sections containing normal and malignant cells (which previous antibodies did not do). Optimization of conditions for staining will follow. We anticipate this Task will be complete within 2-4 months.

Aim 2. Perform an immunohistological analysis of Bin1 in staged primary tumors and metastases.

Task 1 to gather, catalog, and grade 30 frozen tumors was completed. Task 2 to accumulate fixed tumor sections of different grades is pending completion of the antibody characterization for fixed tissue immunohistochemistry. However, this task will not take long to complete, when ready, as Dr. Tomaszewski has already cataloged a significant number of tumors and we have identified a commercial source of tissues with follow-up data available for limited cost. Tasks 3 and 4 to perform immunohistochemistry are partially completed and now published (Ge *et al.* 2000). We examined 30 cases of frozen primary prostate cancer. 29/30 were positive for nuclear staining of Bin1. Scoring of nests of malignant cells indicated that the level of Bin1 in malignant cells in the sections were actually slightly higher than in normal cells, for reasons that were unclear. The increase was at best 2-fold but statistical analysis of the scoring, which was done double blind by our collaborators Drs. Minhas and Tomaszewski, indicated it was significant. Unfortunately, as is the case now for cases of frozen prostate cancer, there were no metastatic variants of the tumors examined from patients to determine whether Bin1 levels were reduced during progression. We believe this is likely, however, given that Northern analysis indicates undetectable levels of Bin1 in 10/10 metastatic lesions (Ge *et al.* 2000) that were kindly provided by Dr. Peter Nelson (CaPCURE Tissue Consortium, University of Washington). Continuation of the immunohistochemical analysis of fixed archival material, where follow-up data are available, awaits completion of antibody characterization.

Aim 3. Identify mutations in the Bin1 gene in androgen-independent tumor cells.

Tasks 1-4 to analyze the state of the Bin1 gene in prostate cancer cells is essentially complete. We examined DNA isolated from 23 microdissected tumors provided by Dr. Tomaszewski to determine if they were heterozygous for a microsatellite marker that we identified in intron 5 of the human Bin1 gene. 19 tumors gave interpretable data and 15/19 were informative for the marker. 6/15 of the informative tumors exhibited loss of heterozygosity (LOH), giving a rate of 40% loss of the Bin1 gene. This finding was in excellent agreement with a study that identified LOH at a rate of 42% in the midsection of chromosome 2q, where Bin1 is located. All coding exons expressed in normal prostate cells were examined for mutations, at the remaining allele in the 6/15 tumors exhibiting LOH. We found no clear evidence of mutation. A potential polymorphism was possible (depending on splice site selection) at the exon 2-3 border, leading to L52Q alteration. All six tumor DNAs exhibited this feature making its significance unclear. These tumors were all primary tumors and therefore likely to be androgen-dependent. Analysis of androgen-independent tumors is pending.

In the meantime, we have completed a thorough examination of Bin1 status in the androgen-dependent cell line LNCaP and the androgen-independent cell lines PC3 and DU145. LNCaP is normal, expressing a normal level of wild-type Bin1 messages (-10 and -10-13 isoforms (Wechsler-Reya *et al.* 1997)). In contrast, PC3 and DU145 each expressed reduced levels of a misspliced polypeptide that is known to be a loss-of-function isoform (Ge *et al.* 1999; Ge *et al.* 2000). The missplice event is inclusion of exon 12A, which normally appears only in neuronal isoforms of Bin1, but has also been seen in melanoma (Ge *et al.* 1999). Although this missplice event is sufficient for loss of function PC3 also sustains a mutation in this polypeptide. In summary, both PC3 and DU145 have sustained loss of function in Bin1 whereas the androgen-dependent LNCaP cells have normal Bin1 function intact.

Aim 4. Investigate the apoptotic potential of Bin1 in androgen-independent cells.

The research design in this aim has been altered due to our experience that the recombinant adenoviruses to be used exhibited unacceptable levels of nonspecific toxicity in our prostate cancer cell lines. In place of Tasks 1 and 2, we performed a colony formation assay to determine whether ectopic expression of Bin1 affected the growth potential of LNCaP, PC3, or DU145 cells. Bin1 was observed to inhibit colony formation in LNCaP and PC3 cells but had much less effect on DU145 cell growth.

Although inducible cell lines would be desirable to study the effect on LNCaP and PC3 cells in more detail, to date our experience has been that existing systems are too leaky and that stably transfected cells lose the ability to inducibly express the transgene (even in the absence of inducer). We are still attempting to overcome this issue.

In a parallel line of work, we have observed that ectopic expression of Bin1 engages an apoptotic-like cell death process that is caspase-independent (Elliott *et al.* 2000). Briefly, this process is characterized by cell shrinkage, substratum detachment, blebbing and vesiculation, and DNA degradation. Cells do not exhibit signs of necrotic death despite the lack of caspase activation. Moreover, neither Bcl-2 nor inhibition of the Fas death receptor pathway block Bin1-induced cell death. The process is specific at some level, because SV40 T antigen can inhibit cell death by Bin1, and the DNA degradation observed is blocked by the serine protease inhibitor AEBSF (which also inhibits apoptosis by c-Myc (Kagaya *et al.* 1997)). The T antigen connection is interesting, insofar as Bin1 can kill cells that lack p53 or Rb function, suggesting that T antigen interferes with Bin1 function at some level other than affecting p53 or Rb function. While the mechanism is yet unclear, we have developed a Bin1 "knockout" mouse model the study of which is providing a useful model to further unravel the mechanism of Bin1-dependent cell death.

We are altering Aim 4 to incorporate use of this 'knockout' system. One goal is to determine whether loss of Bin1 in prostate tissue promotes prostate tumor formation and/or progression. Prostate models are currently limited. Using a published method (Zhang *et al.* 2000), we aim to generate a prostate-specific Myc oncomouse and cross these mice to the Bin1 mice. The Myc oncomouse generated has been shown to progress to prostatic intraepithelial neoplasia (PIN) but not farther (R. Buttyan, Columbia University, pers. comm.). Our prediction is that loss of Bin1 will promote progression, possibly corroborating the role of Bin1 loss at later stages of prostate cancer development and providing a model for prostate tumorigenesis (which are greatly desired). A second goal of work in the 'knockout' system is mechanistic analysis. Preliminary work with Bin1 null fibroblasts has demonstrated that loss of Bin1 renders cells resistant to apoptosis by TNF, DNA damage, and other insults. Current effort to focus on the defective response to TNF, the signaling pathways of which have been worked out at a high level of detail, will greatly promote efforts to determine how Bin1 promotes apoptosis and how its loss may be important in prostate cancer development (where there is evidence that progression to TNF resistance parallels the progression to androgen independence)

Conclusions

We have developed a new set of antibodies that may be useful in pathological analysis of fixed tissues. Analysis of frozen tissues has revealed that Bin1 losses occur primarily at later stages of prostate cancer progression concomitant with the acquisition of metastatic potential. Genetic evidence of LOH in primary tumors is not associated with mutation at the remaining allele. Gene status in metastatic tumors remains to

be examined. However, Northern analysis of such tumors revealed universal loss of Bin1 expression. Consistent with loss of Bin1 function in advanced tumors that have progressed to androgen-independence, we found that androgen-dependent LNCaP cells expressed normal Bin1 but that androgen-independent PC3 and DU145 cells sustained a loss-of-function due to missplicing. Continuing studies of the consequences of Bin1 expression in malignant cells and the mechanism by which Bin1 blocks the growth of prostate cancer cell lines has focused on use of a newly available Bin1 "knockout" mouse. Recent data indicates that loss of Bin1 elicits resistance to a variety of apoptotic stimuli relevant to prostate cancer progression, including resistance to TNF and DNA damage.

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Publications and manuscripts related to this grant (see Appendices for preprints and reprints)

1. Ge, K., Minhas, F., DuHadaway, J., Mao, N.-C., Wilson, D., Sakamuro, D., Buccafusca, R., Nelson, P., Malkowicz, S.B., Tomaszewski, J.T. and Prendergast, G.C. (2000). Loss of heterozygosity and tumor suppressor activity of Bin1 in prostate carcinoma. Int. J. Cancer **86:** 155-161.

   This is our first but fairly extensive study of Bin1 status in prostate cancer.

2. Elliott, K., Ge, K., Du, W., and Prendergast, G.C. (2000). The c-Myc-interacting protein activates a caspase-independent cell death process. Oncogene, in press.

   This study presents an initial investigation of the mechanism of Bin1 action in malignant cells

3. Elliott, K., Sakamuro, D., Basu, A., Du, W., Wunner, W., Steller, P., Gaubatz, S., Zhang, H., Prochownik, E., Eilers, M., and Prendergast, G.C. (1999). Bin1 functionally interacts with Myc and inhibits cell proliferation through multiple mechanisms. Oncogene **18:** 3564-3573.

   This study presents assays to analyze functional significance of Bin1 alterations identified in CaP

4. Mao, N.-C., Steingrimsson, E., Duhadaway, J., Wasserman, W., Ruiz, J., Copeland, N.G., Jenkins, N.A., and Prendergast, G.C. (1999). The murine Bin1 gene functions early in myogenic differentiation and defines a novel region of synteny between human chromosome 2 and mouse chromosome 18. Genomics **56:** 51-58.

   This paper offers the structure of mouse Bin1 gene leading to generation of the knockout mouse

5. Sakamuro, D., Duhadaway, J., Ewert, D., Crouch, D.H., and Prendergast, G.C. A necessary role for Bin1 in c-myc-mediated apoptosis. Manuscript under revision.

   Initial evidence implicating Bin1 in c-Myc-driven apoptosis. The findings suggest why Bin1 may be lost in tumor cells that overexpress c-Myc, like prostate cancer cells.

Presentations describing Bin1 in prostate cancer were made at the following meetings in 1998-99:

a. Annual Oncogene Meeting, June 1999, Frederick MD - poster presentation
b. 1999 90th Annual Meeting of the American Association for Cancer Research, Philadelphia PA
c. CaPCURE Meeting, September 1998, Incline Village NV - poster presentation

Personnel supported by this grant

George C. Prenderast, Ph.D.	Associate Professor
Daitoku Sakamuro, Ph.D.	Research Associate
Ai-Xue Liu, Ph.D.	Research Associate

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Publication of the International Union Against Cancer

LOSS OF HETEROZYGOSITY AND TUMOR SUPPRESSOR ACTIVITY OF *BIN1* IN PROSTATE CARCINOMA

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The genetic events underlying the development of prostate cancer are poorly defined. c-Myc is often activated in tumors that have progressed to metastatic status, so events that promote this process may be important. Bin1 is a nucleocytoplasmic adaptor protein with features of a tumor suppressor that was identified through its ability to interact with and inhibit malignant transformation by c-Myc. We investigated a role for Bin1 loss or inactivation in prostate cancer because the human *Bin1* gene is located at chromosome 2q14 within a region that is frequently deleted in metastatic prostate cancer but where no tumor suppressor candidate has been located. A novel polymorphic microsatellite marker located within intron 5 of the human *Bin1* gene was used to demonstrate loss of heterozygosity and coding alteration in 40% of informative cases of prostate neoplasia examined. RNA and immunohistochemical analyses indicated that *Bin1* was expressed in most primary tumors, even at slightly elevated levels relative to benign tissues, but that it was frequently missing or inactivated by aberrant splicing in metastatic tumors and androgen-independent tumor cell lines. Ectopic expression of *Bin1* suppressed the growth of prostate cancer lines *in vitro*. Our findings support the candidacy of *Bin1* as the chromosome 2q prostate tumor suppressor gene.
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The genetic causes of prostate cancer remain largely unknown. While common in men, prostate tumors are generally both localized and indolent, so genetic events that predispose or promote conversion to an aggressive, metastatic status are of particular interest. One of the most common chromosomal abnormalities seen in tumors that have acquired invasive and metastatic potential is gain of chromosome 8p, where the c-*Myc* gene is located (Bova and Isaacs, 1996). Gains of 8q are well-correlated with disease progression insofar as they are found in 85% of lymph node metastases and in 89% of recurrent hormone refractive tumors (Cher *et al.,* 1996; van den Berg *et al.,* 1995; Visakorpi *et al.,* 1995). c-*Myc* amplification or overexpression is found in many prostate tumors and is a likely progression marker (Buttyan *et al.,* 1987; Fleming *et al.,* 1986; Jenkins *et al.,* 1997). Oncogenic activation of c-Myc by gene amplification delivers a powerful signal that is sufficient to drive cell cycle progression and malignant growth in many types of cells, including prostate cells (Thompson *et al.,* 1989). However, in premalignant cells, c-Myc can also activate apoptosis such that its oncogenic activation is balanced by apoptotic penalty (Prendergast, 1999). Therefore, malignant cells may escape this penalty by inactivating tumor suppressor functions. p53 is an important player but is likely irrelevant to this process in prostate cells because inactivation of p53 does not compromise c-Myc-mediated apoptosis in epithelial cells (Sakamuro *et al.,* 1995; Trudel *et al.,* 1997). Thus, loss or inactivation of molecules other than p53 should be considered.

c-Myc lies at an intersection of 2 signaling networks that target its C-terminal DNA binding domain and its N-terminal transcriptional transactivation domain (Sakamuro and Prendergast, 1999). The major player in the C-terminal network is Max, a helix-loop-helix/"leucine zipper" (HLH/Z) protein that heterodimerizes with c-Myc and mediates its ability to specifically recognize DNA. However, Max alterations do not occur in any human cancer, including prostate cancer. The Max-binding protein Mxi1, which can titer Max away from c-Myc, has been reported to be altered in prostate tumors, but this event appears to represent at best only a fraction of tumors where c-Myc is overexpressed. Recent advances have led to the identification of a set of novel N-terminal-interacting proteins that constitute a second Myc network (Sakamuro and Prendergast, 1999). One of these proteins, Bin1 (Bridging INtegrator 1), is a ubiquitous adaptor protein that has features of a tumor suppressor (Elliott *et al.,* 1999; Sakamuro *et al.,* 1996) and that has been linked to cell death and differentiation decisions (Prendergast, 1999; Sakamuro and Prendergast, 1999). We hypothesized that Bin1 might be inactivated in prostate cancer because the human *Bin1* gene maps to chromosome 2q14 (Negorev *et al.,* 1996), within a region of chromosome 2q that is frequently deleted in metastatic prostate tumors (Cher *et al.,* 1996) but where no tumor suppressor gene has been identified to date. Since c-*Myc* is frequently amplified in prostate carcinomas and Bin1 can suppress malignant transformation by c-Myc (Sakamuro *et al.,* 1996), loss of Bin1 activity would eliminate a mechanism that can limit full oncogenic activation of c-Myc. Our results suggest that loss or inactivation of the *Bin1* gene occurs frequently in prostate cancer, in support of its candidacy for the 2q prostate tumor suppressor gene.

MATERIAL AND METHODS

LOH analysis

Normal lymphocyte genomic DNAs were a gift from H. Reithman (Philadelphia, PA). Genomic DNAs were isolated by standard methods from microdissected malignant and adjacent normal tissues obtained from the Department of Urology at the University of Pennsylvania (Philadelphia, PA). For PCR analysis, an approximately 141 bp fragment containing a $(TG)_{17}$ microsatellite identified in intron 5 was amplified by 35 cycles of PCR. Oligonucleotide primers were 5′-TTTCTGAGGCAGGCTTCCCACTTC and 5′-CGTCTGTGTGAAGAGGTGTGTG. Reactions were per-

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formed in 50 μl and contained 60 mM TrisCl pH 10/1.5 mM $MgCl_2$/15 mM NH_4SO_4/1 mM dNTPs/1U Taq polymerase including 1 μl 800 mCi/mmol [^{32}P]-dCTP. The PCR cycle was 1 min at 94°C/30 sec at 54°C/30 sec at 72°C. Reaction products (4 μl) were examined on agarose gels to verify the expected product and then fractionated on a standard 6% sequencing gel, dried and autoradiographed.

Northern analysis

Prostatic tissues were obtained through the CaPCURE Tissue Consortium at the University of Washington (Seattle, WA). Total cytoplasmic and total cellular RNAs were isolated from cell lines and tissues and analyzed by Northern blotting. Briefly, 10 μg RNA per lane was fractionated on 1.0% agarose gels, blotted to Duralon membranes (Stratagene, La Jolla CA) and hybridized to human *Bin1* or c-*Myc* cDNA probes.

RT-PCR

One microgram total cytoplasmic RNA in 5 μl water was denatured by a 5-min incubation at room temperature with 2 μl 0.1 M methylmercury hydroxide. To this RNA were added 2.5 μl 0.7 M β-mercaptoethanol and 0.5 μg random hexanucleotides in 1 μl aqueous solution. Following a 2-min incubation at 70°C, RNAs were incubated on wet ice, and 2.0 ml 5 μM dNTPs, 0.5 μl RNase inhibitor (Promega, Madison WI), 4 μl 5× RT buffer, 2 μl 0.1 M DTT and 1.0 μl Mo-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) were added. Reactions were incubated 60 min at 37°C and then stopped by a 5-min incubation at 95°C. This RT product was used as template to amplify *Bin1* or β-actin cDNA by PCR. For *Bin1* (exons 1–5), the 5′ primer was 5′-AAAGATCGCCAGCAACGTGC and the 3′ primer was 5′-CTGGTGGTAATCCATCCACAGC. For β-actin (exons 3–4), the 5′ primer was 5′-GGTGGGCATGGGTCAGAAGG and the 3′ primer was 5′-GCAGCTCGTAGCTCTTCTCC. Reactions were performed in 100 μl containing 200 ng template, 50 pmol each primer, 0.4 mM each dNTP, 1× Taq PCR buffer (BMB) and 2.5 units Taq polymerase. PCR was performed by a 2-min incubation at 96°C and then 26 cycles (*Bin1*) or 22 cycles (β-actin) of 30 sec at 96°C/45 sec at 61°C/45 sec at 72°C followed by a final extension of 10 min at 72°C. Products were purified from 1.4% TBE agarose gels and in some cases subjected to direct DNA sequencing. For RNA samples in which the entire coding region of *Bin1* cDNA was cloned by RT-PCR and subjected to direct DNA sequencing, 3 separate PCR reactions were performed using primers and conditions that have been described (Wechsler-Reya *et al.,* 1997*b*).

Immunohistochemistry

Tissue obtained from 30 radical prostetectomy specimens of patients undergoing surgery at the Hospital of the University of Pennsylvania was used. The radical prostectomy specimens were inked and serially sectioned, and areas grossly suspicious for prostatic adenocarcinoma and adjacent benign tissue were taken. Frozen sections were immediately obtained or the tissues were stored at −70°C after snap-freezing in liquid nitrogen. The tumors were classified using the Gleason grading system. In addition, in each section, prostatic intraepithelial neoplasia (PIN) was identified, and benign prostatic tissue was subcategorized where appropriate as benign prostatic hyperplasia (BPH) or atrophy. Frozen tissue sections were warmed to room temperature, washed twice with PBS, incubated in 4% paraformaldehyde/PBS for 30 min at 4°C, rinsed in water and then twice more with PBS. Tissues were permeabilized in 0.1% Triton-X-100 for 10 min, washed twice again with PBS and incubated for 15 min in 1% H_2O_2 in methanol to quench endogenous peroxidase. After washing in PBS and then in PBS/0.1% BSA for 5 min, samples were incubated for 30 min with anti-Bin1 monoclonal antibody (MAb) 99D (Wechsler-Reya *et al.,* 1997*a*), washed twice in PBS and incubated an additional 30 min with the secondary antibody diluted at 1:200 in PBS/0.1% BSA without sodium azide (Biotin-SP-conjugated AffiniPure Goat Anti-Mouse IgG(H+L), Jackson ImmunoResearch, Rockland, PA, 115-065-062). To develop the slides, samples were incubated 30 min with tertiary reagent diluted at 1:200 in PBS/0.1% BSA without azide (peroxidase-conjugated streptavidin, DAKO, Carpinteria, CA, P-0397), washed in PBS 3 times for 5 min, then the slides were flooded 5 min with substrate (Peroxidase Substrate Kit DABkit, Vector, Burlingame, CA, SK-4100). After rinsing in water, slides were counterstained with 0.04% light green in acidified water or dilute hematoxylin for approximately 1 min, dehydrated and cover-slipped. 99D efficiently stained sections from frozen tissue but not tissues that were fixed before sectioning.

Cell culture

LNCaP, PC3 and DU145 cells were obtained from ATCC. Cells were cultured in DMEM containing 10% FCS (Life Technologies) and penicillin/streptomycin (Fisher, Pittsburgh, PA). Cells were transfected using a standard calcium phosphate precipitation method. Briefly, 2×10^5 cells seeded in 10-cm dishes were transfected overnight (18 hr) with 20 μg empty pcDNA3.1 vector (Invitrogen, La Jolla, CA) or the human *Bin1* vector CMV3-Bin1 (Elliott *et al.,* 1999). The next day, cells were washed and refed with growth media. After an additional 24 hr, cells were trypsinized and passaged at 1:3 or 1:6 or into new dishes, and the following day G418 was added to 0.5 mg/mL for selection of stable transfectants. Colonies were scored by methanol fixation and crystal violet or Coomassie blue staining 2–3 weeks later.

RESULTS

Loss-of-heterozygosity (LOH) analysis was performed with genomic DNAs isolated from a set of malignant and patient-matched normal prostate tissues as follows. The marker was a novel dinucleotide microsatellite $(TG)_{17}$ sequence that was identified in intron 5 of the human *Bin1* gene during its characterization (Wechsler-Reya *et al.,* 1997*b*). Examination of this sequence in genomic DNAs isolated from the peripheral blood lymphocytes of 12 unrelated individuals indicated the existence of several alleles. The informative content of the marker indicated utility for LOH analysis insofar as heterozygosity was observed in 10/12 (83%) of the samples examined (Fig. 1*a*). Genomic DNAs isolated from

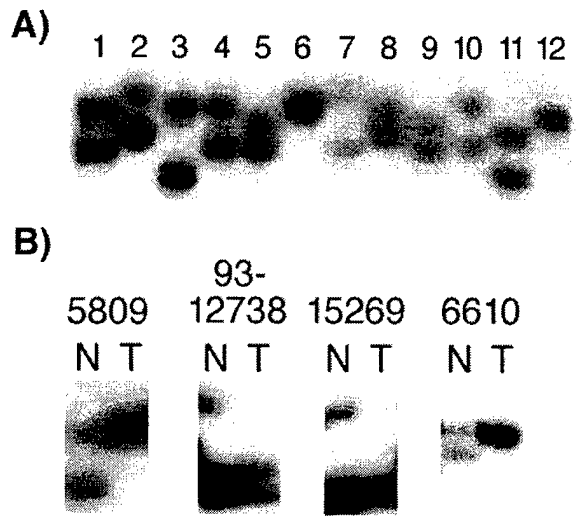

FIGURE 1 – Allelic deletion of Bin1 in prostate cancer. (*a*) Definition of a polymorphic microsatellite within intron 5 of the human *Bin1* gene. PCR products were checked on agarose gels to verify the expected size and abundance and then fractionated on a 6% sequencing gel and autoradiographed. (*b*) Examples of LOH in prostate tumor DNAs. Genomic DNAs isolated from microdissected malignant tissues and matched normal prostate tissues were subjected to PCR and gel fractionation as above. Representative results are shown from 4 informative pairs exhibiting LOH.

cells microdissected from 19 mid-range Gleason score tumor and adjacent normal tissues were analyzed for heterozygosity of the marker (Table I, Fig. 1*b*). In this panel, 15/19 pairs were informative for the marker, and 6/15 (40%) of the informative pairs exhibited LOH. The allelic losses observed were specific because a similar analysis of 18 informative bladder cancer DNAs showed no cases of LOH (Table I). The frequency of LOH in prostate cancer was in excellent agreement with the results of Cher *et al.* (1996), who identified deletions at mid-2q with a frequency of 42%. The DNA sequence of the 15 non-neuronal exons of the remaining allele was determined in the 6 samples that exhibited LOH. A consistent and nonconservative coding region alteration was observed in 5/6 of these samples at a residue that was evolutionarily conserved (L52Q) and located in the functionally important N-terminal BAR region (Elliott *et al.*, 1999). The similarity of this variation was not due to cross-contamination of the samples because other alterations were present that did not affect coding potential (*i.e.*, at wobble bases in the codon) but which were unique to each individual sample. Analysis of genomic DNAs from other malignant prostate tissues revealed cases of similar alterations (data not shown), suggesting the possibility of polymorphism. In any case, the frequency and occurrence of this alteration within a functionally crucial region of *Bin1* supported the possibility that malignant phenotypes may be affected at some level.

We next examined the status of *Bin1* expression by performing Northern, RT-PCR and immunohistochemical analyses of a panel of benign, malignant and metastatic prostate tissues and of the prostate cancer cell lines LNCaP, PC3 and DU145. Northern analysis of benign prostate tissues showed that RNA levels were similar to those seen in placenta (a positive control tissue), consistent with previous observations that suggest ubiquitous expression of this gene (Sakamuro *et al.*, 1996). Primary tumors exhibited relatively similar or modestly reduced levels of Bin1 message when normalized to levels of 18S ribosomal RNA (Fig. 2*a*). An exception was seen in sample 2017R, where Bin1 message was undetectable. In contrast, both metastatic lesions examined in this panel lacked detectable Bin1 RNA (Fig. 2*a*). This observation was confirmed and extended by analysis of a second set of metastatic tumors, which suggested that losses of *Bin1* at this stage of progression occurred frequently (Fig. 2*b*). Hybridization of the same Northern blot with a c-*Myc* probe showed that *Bin1* losses occurred in 4/7 metastatic lesions that overexpressed c-*Myc* but also in the remaining lesions that lacked this event (Fig. 2*b*). The extent of such losses therefore extended broadly, a finding consistent with evidence from structure-function studies indicating that Bin1 has both Myc-independent and Myc-dependent tumor suppressor properties (Elliott *et al.*, 1999).

To examine Bin1 protein levels in prostate cancer, we performed an immunohistochemical analysis of 30 cases of mid-Gleason-grade primary prostate tumors that included tumorous and benign tissues (Fig. 3, Table II). This analysis was conducted with anti-Bin1 MAb 99D, which stains frozen but not fixed tissues, limiting the analysis to primary tumors. Stroma and atrophic cells characteristic of aging tissues stained weakly relative to epithelial cells in benign prostatic hyperplasia (BPH), which were uniformly positive (Fig. 3*ad*) in the same fashion as epithelial cells in normal tissues (data not shown). The nuclear staining pattern observed was consistent with previous studies of Bin1 localization in tissues and tissue culture cells. Prostatic adenocarcinoma and prostatic intraepithelial neoplasia (PIN), a preneoplastic lesion, also exhibited positive nuclear staining (Fig. 3*e–h*). Nuclear counts indicated that cancerous cells stained slightly more strongly than BPH cells (Table I). Statistical analysis of these data established that the difference between cancerous and BPH and especially atrophic cells was statistically significant (Fig. 4). The trend in the immunohistochemical results, while modest, differed from that seen by Northern analysis, possibly reflecting the effects of tumor heterogeneity (which is quite significant in prostate malignancy) or differences in protein stability. Nevertheless, the immunochemical results clearly indicated that *Bin1* is expressed in benign as well as

TABLE I – LOSS OF HETEROZYGOSITY ANALYSIS

Tissue type	Informative samples	Allelic losses	Percent LOH
Prostate	15	6	40
Bladder	18	0	0

Genomic DNAs isolated from a set of tumorous and patient-matched benign tissues were analyzed for heterozygosity of a dinucleotide microsatellite $(TG)_{17}$ sequence located in intron 5 of the human *Bin1* gene (Wechsler-Reya *et al.*, 1997*b*). Prostate tumors were mid-range Gleason-grade primary adenocarcinomas. DNAs isolated from bladder tissues were used as a control to address the specificity of allelic losses.

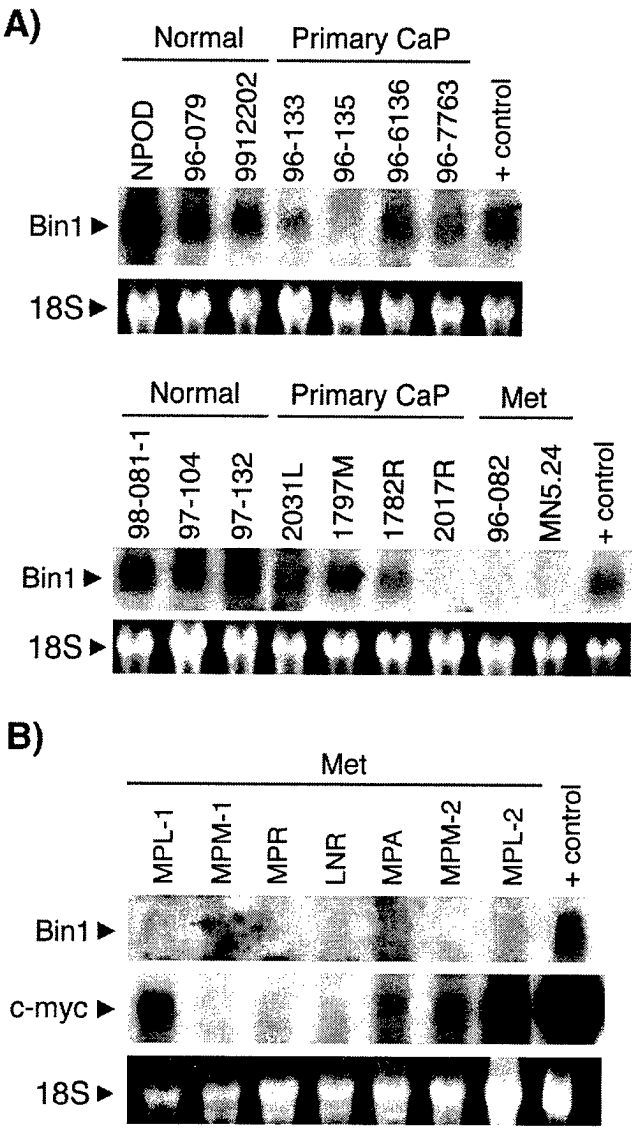

FIGURE 2 – Frequent losses of Bin1 message in metastatic prostate tissues. (*a*) Bin1 expression in a panel of normal, primary tumor and metastatic human prostate tissues. Total RNAs isolated from tissue samples indicated were subjected to Northern analysis using a *Bin1* cDNA probe (Sakamuro *et al.*, 1996). Placental RNA was used as a positive control for detection of Bin1 RNA. Met: metastatic tumors. (*b*) Losses in metastatic tumors are frequent and extend to tumors where c-Myc is not overexpressed. The Northern blot was sequentially hybridized with *Bin1* and human c-*Myc* cDNA probes to identify tumors where c-Myc was overexpressed.

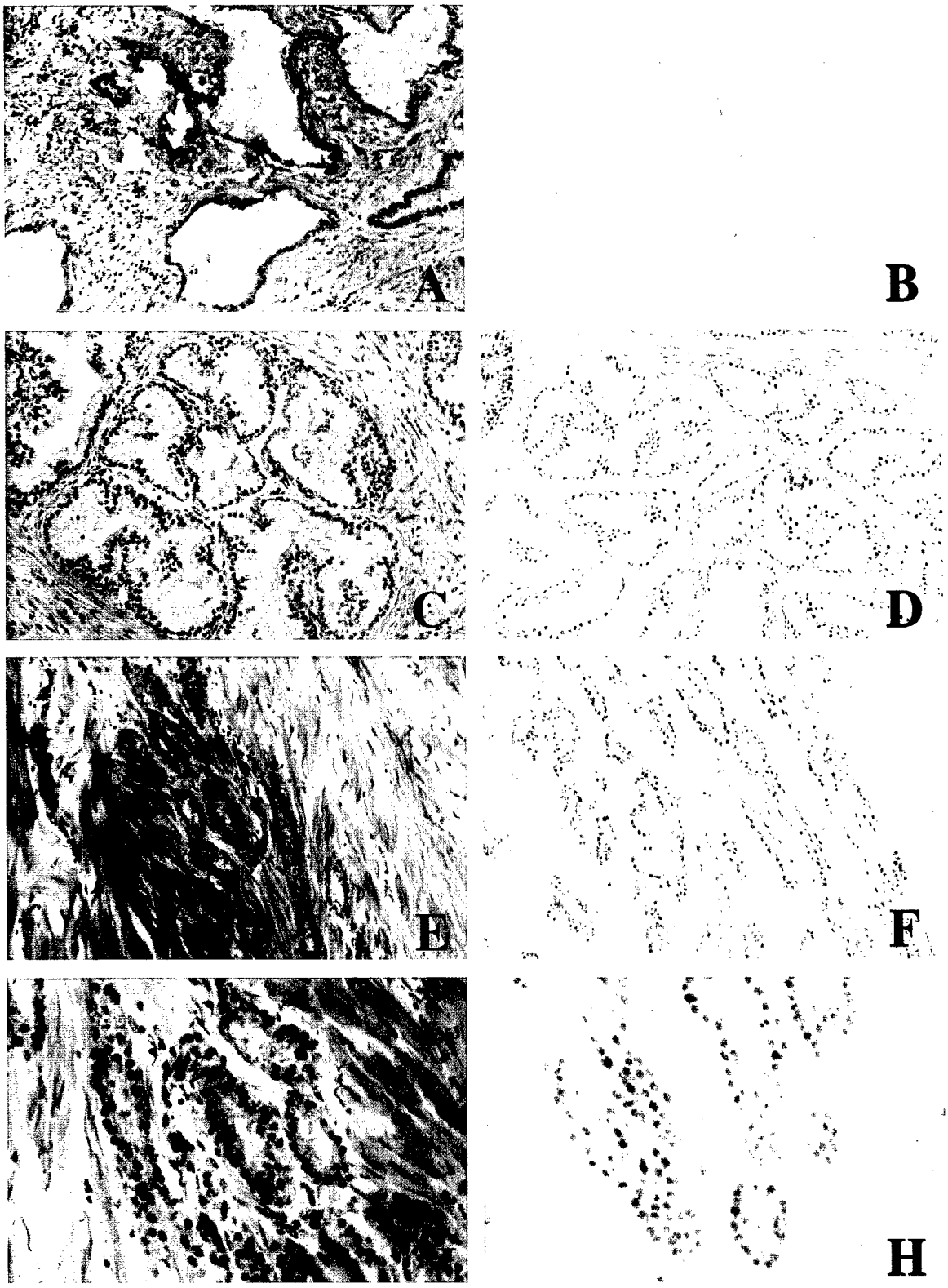

FIGURE 3 – Immunohistochemical analysis. Thirty frozen cases of primary prostate adenocarcinoma that included normal tissue, benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN) and prostatic atrophy were subjected to immunohistochemistry with the anti-Bin1 MAb 99D (Wechsler-Reya *et al.*, 1997*a*). Adjacent sections were stained with H&E (*a,c,e,g*) or processed for 99D staining (*b,d,f,h*). Stromal cells stained lightly for the Bin1 antigen(s) under the conditions employed. The quantitative results of staining intensities observed in each tissue are presented in Table II and Figure 4. (*a,b*) Atrophy. Little to no staining is evident. (*c,d*) Benign hyperplasia. Epithelial cell nuclei are stained to a degree similar to that seen in a variety of normal tissues including prostate. (*e,f,g,h*) Prostatic adenocarcinoma. Nuclear staining is retained in both PIN and primary malignant cells.

TABLE II – IMMUNOHISTOCHEMICAL ANALYSIS[1]

Case number	Gleason grade	CaP		BPH		PIN		Atrophy	
		Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei	Bin1 score	Number of positive nuclei
1	5+4	3+	130/500	2+	70/500				
2	3+4	4+	410/500						
3	3+3	4+	460/500						
4	3+4	3+	455/500	2+	205/500	3+	420/500	1+	60/500
5	4+4	4+	380/500	2+	245/500			1+	10/500
6	3+3	4+	405/500	1+	190/500				
7	3+3	4+	435/500	3+	365/500				
8	3+4	4+	480/500	2+	260/500			1+	70/500
9	3+4	4+	418/500	3+	360/500				
10	3+3	4+	398/500	3+	265/500				
11	4+3	4+	432/500	3+	370/500				
12	2+3	4+	322/500	3+	290/500			1+	70/500
13	3+3	4+	446/500	1+	50/500			1+	30/500
14	3+3	4+	480/500	3+	390/500				
15	4+5	3+	385/500	3+	310/500				
16	3+3	3+	385/500	3+	345/500			1+	85/500
17	3+3	3+	390/500	2+	290/500			1+	80/500
18	3+3	4+	410/500	3+	355/500	3+	385/500		
19	3+4	4+	392/500	1+	155/500	4+	343/500	1+	25/500
20	3+3	3+	380/500	1+	60/500				
21	3+3	4+	320/500	3+	210/500			1+	80/500
22	3+4	3+	415/500	3+	317/500			1+	35/500
23	3+4	0+	05/500	1+	50/500				
24	3+4	4+	430/500	2+	170/500			1+	25/500
25	3+3	4+	351/500	2+	170/500	3+	305/500	1+	23/500
26	3+4	4+	358/500	2+	160/500	3+	240/500	1+	30/500
27	3+3	4+	442/500	2+	195/500			1+	18/500
28	3+3	3+	365/500	3+	320/500			1+	35/500
29	3+3	4+	357/500	3+	300/500	4+	350/500	1+	60/500
30	3+3	4+	354/500	2+	210/500	3+	295/500	1+	40/500

Frozen tissues obtained from 30 radical prostetectomy specimens of patients undergoing surgery for prostatic adenocarcinoma were examined. Tissue sections examined included both benign and tumor tissue. Slides were scored by 2 observers based on the mean extent of nuclear Bin1 staining, with 500 cells of each tissue type counted. Zero (0) was assigned for no positivity of cells in the section; 1 for <2% positivity; 2 for 2%–10% positivity; 3 for 10%–50% positivity and 4 for >50% positivity of tumor, PIN or BPH as appropriate. For statistical analysis, only those cases with a score of 3 or above were considered positive, with the remainder classified as negative or weak. The ANOVA algorithm in Stat View V1.03 software was used for statistical analysis of the results. CaP: cancer of the prostate; BPH: benign prostatic hyperplasia; PIN: prostatic intraepithelial neoplasia; atrophy: atrophic or aging cells sometimes mistaken for malignant cells in tissue biopsy sections.

[1]For $p < 0.05$: CaP *vs.* BPH: significant; CaP *vs.* PIN: not significant; CaP *vs.* atrophy: significant; BPH *vs.* PIN: not significant; BPH *vs.* atrophy: significant; PIN *vs.* atrophy: significant.

primary tumor cells at the protein level, suggesting that losses and/or inactivation may occur mainly in metastatic tumors.

Additional support for this conclusion was provided by an examination of Bin1 structure and expression in the human prostate cancer cell lines LNCaP, DU145 and PC3. LNCaP cells expressed the highest steady-state levels of message, but Bin1 RNA was also detectable in PC3 and DU145 (Fig. 5, lower panels). RT-PCR and DNA sequencing was performed to examine coding region structure and splice patterns because the *Bin1* gene is subjected to tissue-specific splicing (Wechsler-Reya *et al.*, 1997*b*). This analysis showed that androgen-dependent LNCaP cells express the c-Myc-binding Bin1 isoform, which includes exon 13. This exon is constitutively alternately spliced in all normal cells and encodes approximately half of the c-Myc-binding domain. In contrast to LNCaP cells, the androgen-independent PC3 and DU145 cells poorly expressed exon 13 and expressed instead the brain-specific exon 12A (Fig. 5, upper panels). Mis-splicing of exon 12A in the absence of the other brain-specific exons 12B–12D, as seen in PC3 and DU145, has been shown in melanoma to be a nonphysiological and tumor-specific event that abolishes tumor suppressor activity and eliminates the ability of Bin1 to inhibit malignant transformation by c-*Myc* or adenovirus E1A (Ge *et al.*, 1999). Thus, whereas LNCaP cells retain a c-Myc-interacting isoform of Bin1, PC3 and DU145 cells express a Bin1 isoform(s), which lacks the capacity to bind and/or suppress the oncogenic activity of c-Myc. Since acquisition of androgen independence is a characteristic feature of prostate tumors with metastatic capability, the expression of exon 12A isoform(s) in PC3 and

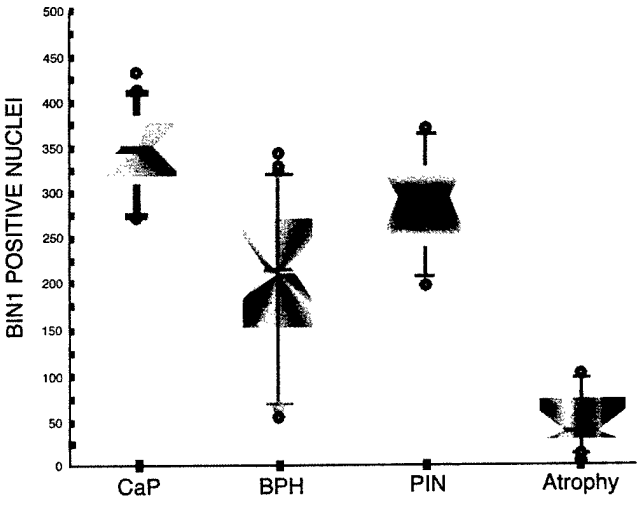

FIGURE 4 – Quantitation of Bin1 levels determined by immunohistochemistry. The distribution and standard error of data shown in Table II were computed and plotted to compare the relative level of Bin1 staining in cells of each pathological type examined. Atrophic cells show a statistically significant suppression in Bin1 levels relative to the other cell types. The < 2-fold increase in Bin1 levels observed in cancerous (CaP) cells relative to BPH cells was also statistically significant in this analysis.

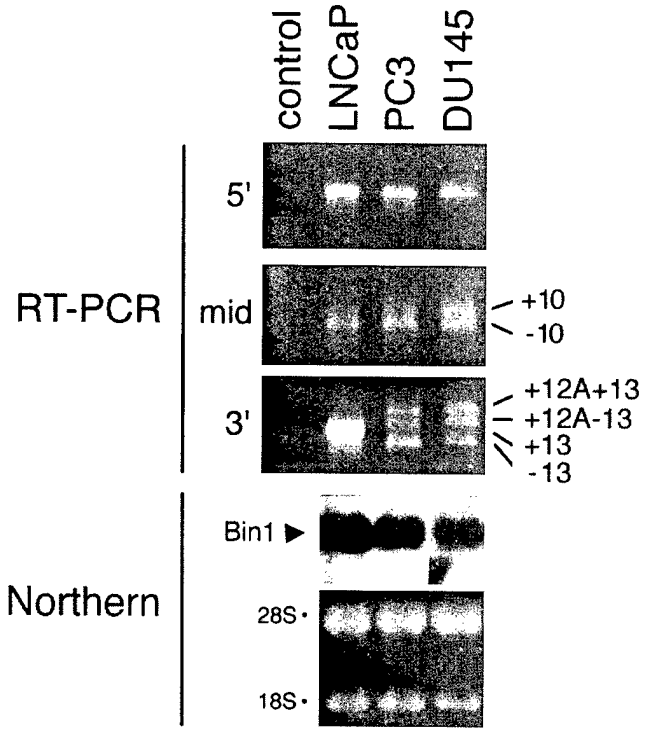

FIGURE 5 – Aberrant splicing of Bin1 in androgen-independent prostate cell lines. Total cytoplasmic RNA isolated from androgen-dependent LNCaP cells and androgen-independent PC3 and DU145 cells were examined by Northern and RT-PCR analyses as described in Material and Methods. The DNA sequence of the complete coding region of each cDNA was determined. Splice pattern and amino acid coding sequences of *Bin1* were wild type in LNCaP. Whereas DU145 had silent alterations but no coding mutations, PC3 included a conservative coding alteration (L214V) within the central U1 region (Elliott *et al.*, 1999). Bottom panels: Northern analysis showing detectable reduction of *Bin1* message in PC3 and DU145 relative to LNCaP cells. Top panels: Results of RT-PCR analysis across 3 separate segments of the cDNA encompassing the complete coding region. Differences in splice isoforms are apparent in the middle (mid) and 3′ regions of *Bin1* cDNA, involving the muscle-specific exon 10 and the brain-specific exon 12A (Wechsler-Reya *et al.*, 1997*b*). Exon 13 encodes part of the c-Myc-binding domain of Bin1. Although constitutively alternately spliced in all normal cells and LNCaP, PC3 and DU145 cells express lower or undetectable levels of the exon 13-containing (13+) isoforms that are capable of c-Myc interaction. Exon 10 splicing in DU145 is an aberrant event but not one that compromises the tumor suppressor activity of Bin1 (Elliott *et al.*, 1999). Splicing of exon 12A in the absence of the brain-specific exons 12B, C and D, which is observed in PC3 and DU145 but not in LNCaP cells, has been shown to be a nonphysiological and tumor-specific event that abolishes the tumor suppressor activity of Bin1 (Ge *et al.*, 1999). Control: RT-PCR performed in the absence of added RNA.

DU145 supports the hypothesis that Bin1 is targeted for loss or inactivation at advanced progression stages, because LNCaP is androgen-dependent whereas PC3 and DU145 are androgen-independent. Ectopic expression of the c-Myc-interacting Bin1 isoform caused a reduction in the relative efficiency of G418-resistant colony formation in PC3 and LNCaP cells but not in DU145 cells (Fig. 6). While the reason for the lack of effect in the latter cell line was unclear, DU145 are resistant to many antiproliferative stimuli, and their resistance provided evidence that Bin1 was not toxic *per se* to prostate cells. In summary, the results supported the conclusion that Bin1 had a negative role in prostate cell growth and was inactivated at some level in prostate cells and that loss or inactivation was likely to have biological significance.

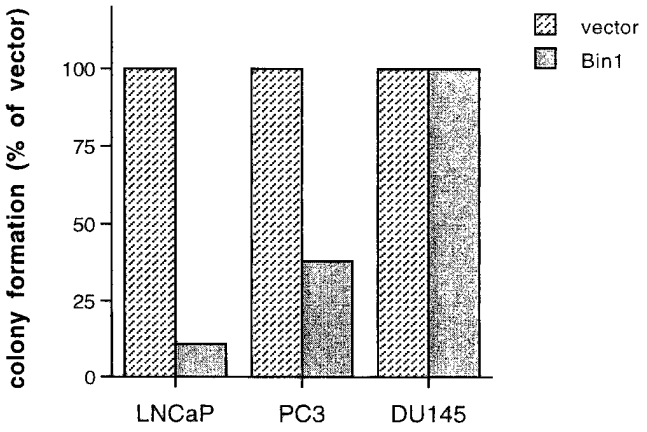

FIGURE 6 – Tumor suppressor activity of Bin1 in prostate cells. Cells were transfected with 20 μg of the neomycin-resistance gene-marked vectors CMV3-Bin1 or pcDNA3 (empty vector), and stable transfectants were selected by culturing in growth media containing G418. Drug-resistant colonies were scored approximately 3 weeks later by fixation in methanol and staining with crystal violet. The colony formation data are presented as the proportion of colonies formed by CMV3-Bin1 relative to those formed by empty vector alone. LNCaP and PC3 exhibit a growth suppression revealed by a reduction in colony formation following ectopic Bin1 expression, whereas DU145 is not affected.

DISCUSSION

Our report identifies the *Bin1* gene as a candidate for the prostate tumor suppressor gene on the middle of the long arm of chromosome 2 (Cher *et al.*, 1996). Allelic deletion and a common alteration was documented at the *Bin1* locus in 40% of a panel of primary prostate tumors examined. We found that LOH in primary tumors was not associated with losses in RNA or protein levels but rather with persistent expression at levels even slightly higher than that seen in benign cells. The basis for the latter was unclear. Alterations of *Bin1* such as the L52Q alteration seen in samples exhibiting LOH might be germane, but their extent and biological consequences, if any, remain to be established. In support of the notion that allelic deletions occur in primary tumors, which are usually androgen-dependent, the androgen-dependent cell line LNCaP has been reported to lack one copy of chromosome 2 (Hyytinen *et al.*, 1997). In contrast to primary tumors, metastatic lesions and androgen-independent cell lines exhibited loss or inactivation of *Bin1*. Losses in metastatic lesions suggest a role for *Bin1* at a later stage in progression when cells acquire the ability to invade and survive in the absence of appropriate adhesion and hormonal signals. The frequency of losses seen extended beyond tumors exhibiting c-Myc overexpression, a feature that may reflect the fact that Bin1 also has c-Myc-independent tumor suppressor activities (Elliott *et al.*, 1999). The inactivation of *Bin1* seen in androgen-independent cell lines occurred by an aberrant splicing event identical to that shown to cause loss of Bin1 tumor suppressor activity in melanoma (Ge *et al.*, 1999). This splice event introduces brain-specific sequences into Bin1, abolishing its ability to suppress malignant transformation by c-Myc or by adenovirus E1A, which acts independently of c-Myc (Ge *et al.*, 1999). Thus, both the c-Myc-dependent and c-Myc-independent tumor suppressor activities of Bin1 are eliminated by aberrant splicing.

The function of Bin1 is complex and involves splice isoform-specific adaptor roles in both the nucleus and cytoplasm (Sakamuro and Prendergast, 1999; Wigge and McMahon, 1998). Nevertheless, there is evidence that the ubiquitously expressed nuclear Bin1 isoform that interacts with c-Myc can modulate transcription mediated by this oncoprotein (Elliott *et al.*, 1999) and can participate in cell death and differentiation decisions that it controls (Prendergast, 1999). Therefore, loss or inactivation of *Bin1* in

cancer may also contribute to the loss of differentiated properties of prostate cells, including their reliance upon androgen and adhesion signals, which occur during malignant progression. Although there is a close association between acquisition of androgen independence and metastatic capability, there is not yet any evidence that Bin1 is regulated by androgens (data not shown). However, Bin1 has been identified also as an integrin-binding protein (Wixler *et al.,* 1999), so it may have a role in integrin signaling to the nucleus. Given opposing trends in the expression of c-Myc and Bin1 in prostate cancer, we favor the notion that Bin1 loss or inactivation may help desensitize cells to apoptosis or differentiation signals, allowing them to amplify c-*Myc* or other oncogenes without apoptotic penalty.

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The c-Myc-interacting adaptor protein Bin1 activates a caspase-independent cell death program

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Abstract

Cell death processes are progressively inactivated during malignant development, in part by loss of tumor suppressors that can promote cell death. The Bin1 gene encodes a nucleocytosolic adaptor protein with tumor suppressor properties, initially identified through its ability to interact with and inhibit malignant transformation by c-Myc and other oncogenes. Bin1 is frequently missing or functionally inactivated in breast and prostate cancers and in melanoma. In this study, we show that Bin1 engages a caspase-independent cell death process similar to type II apoptosis, characterized by cell shrinkage, substratum detachment, vacuolated cytoplasm, and DNA degradation. Cell death induction was relieved by mutation of the BAR domain, a putative effector domain, or by a missplicing event that occurs in melanoma and inactivates suppressor activity. Cells in all phases of the cell cycle were susceptible to death and p53 and Rb were dispensable. Notably, Bin1 did not activate caspases and the broad spectrum caspase inhibitor ZVAD.fmk did not block cell death. Consistent with the lack of caspase involvement, dying cells lacked nucleosomal DNA cleavage and nuclear lamina degradation. Moreover, neither Bcl-2 or dominant inhibition of the Fas pathway had any effect. In previous work, we showed that Bin1 could not suppress cell transformation by SV40 large T antigen. Consistent with this finding, we observed that T antigen suppressed the death program engaged by Bin1. This observation was interesting in light of emerging evidence that T antigen has roles in cell immortalization and human cell transformation beyond Rb and p53 inactivation. In support of a link to c-Myc-induced death processes, AEBSF, a serine protease inhibitor that inhibits apoptosis by c-Myc, potently suppressed DNA degradation by Bin1. Our findings suggest that the tumor suppressor activity of Bin1 reflects engagement of a unique cell death program. We propose that loss of Bin1 may promote malignancy by blunting death penalties associated with oncogene activation.

Introduction

Cell suicide programs are crucial to development and homeostasis. A major role of these programs is to stanch inappropriate cell proliferation that can lead to cancer. Indeed, loss of the capacity for programmed cell death (PCD) is a hallmark of the malignant cell. Loss of this capacity is not due to inactivation of the machinery responsible for the major form of PCD, apoptosis, which is minimally comprised of cytochrome c, apoptosis promoting factors (Apafs), and caspases (Reed *et al.*, 1998). Instead, it appears that malignant cells suppress or eliminate signals needed to commit to PCD and/or to activate the apoptotic machinery.

How cells commit to die and how malignant cells sidestep this decision are questions of great interest in the areas of programmed cell death and cancer research Caspase regulation is important but there is emerging evidence that caspase-independent processes may also have important roles. Death receptors directly activate caspases and cancer cells neutralize these routes by multiple strategies (Ashkenazi & Dixit, 1998). Mitochondria indirectly regulate caspases, by controlling the release of cytochrome c release and thereby the status of Apaf, which controls the activation of caspase-9 (Green & Reed, 1998). This route is blunted in cancer cells primarily by alterations in the level of Bcl-2 family proteins, which control cytochrome c release (Chao & Korsmeyer, 1998; Reed *et al.*, 1998; Thompson & Vander Heiden, 1999), but probably at other levels as well (Ding *et al.*, 1998; Fearnhead *et al.*, 1997). Caspase activation is clearly sufficient for death commitment but whether it is necessary is much less clear. A viable hypothesis is that caspase-independent processes participate in commitment and that caspase activation seals a PCD decision made by the cell (Amarante-Mendes *et al.*, 1998; McCarthy *et al.*, 1997; Thompson & Vander Heiden, 1999). If so, then caspase-independent processes may be disrupted in cancer cells like caspase activation pathways.

One area of investigation into cancer cell death mechanisms centers on how c-Myc stimulates PCD and why it does not do so in malignancy (Prendergast, 1999). Oncogenic activation of c-Myc

promotes the development of many clinically significant cancers, such as those of the breast, colon, lung, and prostate (Cole, 1986; Garte, 1993; Jenkins *et al.*, 1997). c-Myc activation usually occurs at later stages in carcinoma in humans and is usually a poor prognostic marker. However, in premalignant cells c-Myc is a robust stimulator of PCD. Therefore, to exploit the growth-promoting aspects of c-Myc, malignant cells must evolve strategies to escape the death penalty associated its activation. Mechanistic investigations in fibroblast and lymphocyte models have defined central roles for the tumor suppressors p53 and $p19^{ARF}$, which are frequently inactivated in human cancer (Sherr, 1998). Whether these genes mediate PCD by c-Myc or sensitize cells to its action is unclear. Nevertheless, it is evident that p53 inactivation does not compromise the ability of c-Myc to drive PCD in epithelial cells (Prendergast, 1999; Sakamuro *et al.*, 1995), indicating that p53-independent mechanisms are also important. Interestingly, careful investigations have revealed that caspase inhibition or Bcl-2 overexpression does not abolish the ability of c-Myc to commit cells to undergo PCD. Caspase inactivation eliminates nuclear phenotypes characteristic of apoptosis and slows the kinetics of cell death, but it does not abolish all apoptotic phenotypes nor ultimate cellular demise (Amarante-Mendes *et al.*, 1998; Cecconi *et al.*, 1998; McCarthy *et al.*, 1997; Soengas *et al.*, 1999; Yoshida *et al.*, 1998). Similarly, Bcl-2 proteins significantly delay but do not abolish PCD commitment induced by c-Myc either *in vitro* or *in vivo* (McCarthy *et al.*, 1997; Trudel *et al.*, 1997; Tsuneoka & Mekada, 2000). Thus, while c-Myc promotes PCD by activating caspases (Kangas *et al.*, 1998), and Bcl-2 cooperates with c-Myc to promote malignancy by delaying this process, c-Myc apparently also affects caspase-independent processes that influence death commitment. Inactivation of such processes may be important in epithelial cells where c-Myc activation occurs. This may be especially true at stages when malignant cell division is slow and inefficient modes of cell death which do not involve caspases may be effective at stanching tumor outgrowth. Thus, inactivation of caspase-independent processes may contribute to tumorigenesis by helping cells escape death penalties associated with activation of c-Myc or other oncogenes (Prendergast, 1999).

In this study, we investigated a role in PCD for Bin1 (Bridging INtegrator-1), one of an emerging set of c-Myc-interacting adaptor proteins that are candidates for regulating c-Myc or mediating its diverse actions in cells (Sakamuro & Prendergast, 1999). Bin1 function is complex and varied by tissue-specific splicing. It was identified initially through its ability to interact with and inhibit malignant transformation by c-Myc (Sakamuro *et al.*, 1996). Subsequent investigations established that there are two ubiquitous slice isoforms of Bin1 and several other splice forms that are restricted in expression to muscle or brain (Butler *et al.*, 1997; Ramjaun *et al.*, 1997; Tsutsui *et al.*, 1997; Wechsler-Reya *et al.*, 1998; Wechsler-Reya *et al.*, 1997b). Bin1 polypeptides are related in their terminal domains to amphiphysin, a neuronal protein involved in synaptic vesicle endocytosis, and brain isoforms which are most similar have been termed alternately amphiphysin II or amphiphysin-like (amphl). However, amphiphysin was named for its biochemical properties, rather than its function, and outside the brain Bin1 has functions that are not amphiphysin-like. First, although the brain isoforms of Bin1 are cytosolic, the ubiquitous Bin1 isoforms localize to the nucleus as well as the cytosol (Kadlec & Pendergast, 1997; Wechsler-Reya *et al.*, 1997a). Consistent with their nuclear localization, these isoforms functionally interact with the nuclear tyrosine kinase c-Abl as well as with c-Myc (Elliott *et al.*, 1999b; Kadlec & Pendergast, 1997). Second, only brain isoforms of Bin1 include sequences that are required to interact with clathrin and the endocytosis regulatory complex AP-2 (Ramjaun & McPherson, 1998). Third, muscle-specific isoform, which localizes to the nucleus and binds c-Myc, is required for myoblasts to withdraw from the cell cycle and to terminally differentiate (Mao *et al.*, 1999; Wechsler-Reya *et al.*, 1998). Lastly, isoforms that localize to the nucleus and bind c-Myc exhibit tumor suppressor properties which are inactivated or missing in malignant melanoma, breast cancer, and prostate cancer (Ge *et al.*, 1999; Ge *et al.*, 2000a; Ge *et al.*, 2000b). In contrast, amphiphysin and brain isoforms of Bin1 lack suppressor activity. Indeed, one way by which Bin1 is functionally inactivated in cancer is by missplicing of one of its brain-specific exons (Ge *et al.*, 1999). Thus, Bin1 has two functions, one of which is linked to nuclear processes that influence cell fate. In this study, we provide evidence that the tumor suppressor properties of

Bin1 are related to induction of cell death and that Bin1 participates in a caspase-independent process similar to type II apoptosis.

Materials and Methods

Recombinant adenoviruses. Constitutive and inducible adenoviral vectors were developed by standard methods. The constitutive virus Ad-Bin1 contained Bin1 cDNA driven by a cytomegalovirus (CMV) enhancer-promoter in place of the E1 region of the virus. Briefly, the full-length Bin1 cDNA 99fE, that includes exons 1-11 and 13-16 but not brain-specific exons 12A-12D (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997b), was subcloned into pAdCMVlink-1. 293 cells were cotransfected with this derivative plus ClaI-digested dl7001 adenovirus DNA to obtain the recombinant virus by homologous recombination as described (Davis & Wilson, 1996). Similar viral vector constructions used Bin1-10+12A isoform, an aberrant tumor-specific and loss-of-function isoform (Ge *et al.*, 1999), or Bin1ΔBAR-C, a loss-of-function deletion mutant lacking aa 126-207 (Elliott *et al.*, 1999b). Plaque-purified clones were identified by Southern and Western analysis and the DNA sequence of the insert was determined to verify wild-type status. A variation of up to several hours was noted in the kinetics of death induction by different preparations of Ad-Bin1. The inducible virus Ad-MABin1 was constructed similarly in a cre-loxP adenoviral system kindly provided by F.L. Graham (Anton & Graham, 1995) . Briefly, the luciferase cDNA in the vector pMA19 (Anton & Graham, 1995) was replaced with the 99fE cDNA. The resulting plasmid was cotransfected into 293 cells as above to obtain the recombinant virus by homologous recombination. In this virus, the Bin1 cDNA is conditionally expressed under the control of the cytomegalovirus (CMV) promoter, from which it is separated by an loxP-flanked stuffer sequence. Bin1 expression is suppressed in the unrearranged virus because the stuffer region includes stop codons in all three reading frames. The analogous Ad-MA19 virus which expresses luciferase was a gift of F.L. Graham. In cells expressing P1 bacteriophage Cre site-specific recombinase, the intervening stuffer region is removed by Cre-mediated excision, leading to expression of the transgene. Ad-vect is a control adenovirus kindly

provided by J.M. Wilson which is similar in structure to the others except that it contains no transgene (Davis & Wilson, 1996).

Cell culture. HepG2, SAOS-2, and 293 cell lines were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 50 U/mL penicillin and streptomycin (Fisher). For adenovirus infections, HepG2 or SAOS-2 cells were plated at 5×10^5 cells per 6 cm culture dish and allowed to recover overnight. Virus was added to the cells at the indicated m.o.i. in a volume of 1 mL of DMEM supplemented with 2% fetal bovine serum and allowed to adhere for 2 to 3 hours at 37°C. Cells were then washed and fed with DMEM 10% FBS. Where appropriate, the indicated concentrations of the broad spectrum caspase inhibitor ZVAD.fmk (Enzyme Systems Products) was added to cells at the time of infection and maintained until harvesting. Thapsigarin and staurosporine (Calbiochem) were used as chemical inducers of apoptosis at concentrations of 2 μM and 0.5 μM, respectively, and as positive controls produced similar results. For cell viability determinations, cells were harvested 48 hours postinfection by trypsinization, washed in PBS, stained with trypan blue and counted using a hemacytometer. Where indicated, the serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Sigma) or its inactive analog 4-(2-aminoethyl)benzenesulfonamide (AEBSA) (Aldrich), which substitutes an amino group for the crucial fluoride moiety in AEBSF. Both inhibitors were added to cells at infection and maintained at a final concentration of 0.4 mM, a concentration which is nontoxic but sufficient to suppress c-Myc-mediated apoptosis (Kagaya *et al.*, 1997). For electron microscopy, HepG2 cells were infected with Ad-LacZ or Ad-Bin1 (m.o.i. = 100) or treated with 0.5 μM staurosporine and where indicated treated with 100 μM ZVAD.fmk. Adherent and floating cells were harvested 36 hr after adenoviral infection or 24-30 hr after staurosporine treatment and processed for osmium tetroxide staining and electron microscopy using standard methods. Baculovirus experiments were performed in the insect cell line Sf9 using full-length recombinant viruses expressing Bin1, Bcl-2 or no insert, essentially as described (Alnemri *et al.*, 1992; Elliott *et al.*, 1999b). Viable cell counts were determined at the indicated times after virus infection by the trypan blue exclusion method.

Western analysis. Cell lysates was prepared and analyzed by standard protocols (Harlow & Lane, 1988). Briefly, lysates were fractionated by SDS-PAGE gels were electrophoretically transferred to ECL membrane (Amersham) or Immobilon-P (Millipore). Blots were blocked in 3% skim milk and probed with anti-Bin1 monoclonal antibodies 99D, 99I, or α12A or anti-Bcl-2 antibody #124 (DAKO). 99D and 99I recognize epitopes in the c-Myc binding domain encoded by exon 13 whereas α12A recognizes an epitope encoded by brain-specific exon 12A (Wechsler-Reya *et al.*, 1997a; Ge *et al.*, 1999). Antibodies were diluted 1:50 in PBS with 2.5% skim milk and 0.1% Tween-20 and incubated with the membrane 12 hr at 4°C. Blots were washed and incubated 1 hr in the same buffer with secondary goat anti-mouse antibodies conjugated to horseradish peroxidase (BMB) and developed using a chemiluminescence kit using the protocol suggested by the vendor (Pierce).

Immunofluorescence. Cells were seeded on coverslips and the next day infected with recombinant adenoviruses or treated with drugs. After the periods indicated cells were washed twice with PBS, stained 5 min with 5 μg/mL Hoechst 33323 (Sigma) dissolved in PBS, and analyzed immediately by immunofluorescence. For endocytosis assays, cells seeded on coverslips were infected with recombinant adenoviruses and 20 hr later processed for fluid-phase uptake or receptor-mediated endocytosis as described (Barbieri *et al.*, 1998; Benmerah *et al.*, 1998). Briefly, cells were incubated 30 min in serum-free DMEM and then treated with 100 nM fluorescein-conjugated transferrin (Sigma) (receptor-mediated) or 2 mg/mL horseradish peroxidase (Sigma) in 0.2% BSA (fluid-phase). After incubation for 15 min, cells were cooled to 4°C, washed 2X with ice-cold PBS, and fixed in 3.7% paraformaldehyde. Cells were mounted for analysis by confocal fluorescence microscopy or processed with horseradish peroxidase substrate before microscopic analysis.

Flow cytometry. Cells were harvested by trypsinization and fixed in 1% paraformaldehyde and then 80% ethanol. TUNEL assay was performed by incubating samples 1 hr at 37°C in TdT reaction buffer (Boehringher Mannheim) and then staining 30 min at room temperature with

fluoresceinated streptavidin (DuPont). Cells were then incubated 30 min at room temperature with propidium iodide. Alternatively, to analyze only DNA degradation, cells were fixed in 70% ethanol and permeabilized in PBS/0.2% Tween-20 followed by propidium iodide staining as described (Sakamuro *et al.*, 1997). Flow cytometry was performed on a EPIC/XL cell analyzer (Coulter).

Nucleosomal DNA assay. Genomic DNA was prepared by a modified Hirt method (Debbas & White, 1993; Hirt, 1967) or by the following method, as indicated. Cells were gently resuspended in 250 µl TBE buffer (90 mM Tris-borate/2 mM EDTA) containing 0.4% NP40 and 5 µg RNAse A and incubated 30 min at 37°C. Proteinase K and SDS were added to 100 µg/mL and 1% (v/v), respectively, and samples were incubated overnight at 55°C. Genomic DNA was extracted with phenol-chloroform, precipitated with ethanol, and fractionated by agarose gel electrophoresis.

Caspase activity assay. Lysates prepared from equivalent numbers of cells were used to assess caspase-3-like activities, employing the Quantizyme Assay System as recommended by the vendor (Biomol Research Laboratories). Briefly, duplicate plates were harvested 48 hours post-infection, one for cell counts and the other for cell lysates. $5x10^7$ cells/mL were extracted in the cell lysis buffer provided by the vendor and 10 µl of extract was used in reactions to monitor cleavage of the substrate Ac-DEVD-pNA. Reactions were examined at various time after addition of the substrate by spectroscopy at 405 nM to monitor release of the product. Data were analyzed with DeltasoftII Software (Biometallics Inc.).

Results

Bin1 activates a programmed cell death process in malignant cells. We have shown that reintroduction of Bin1 into human cancer cell lines that lack endogenous expression leads to loss of proliferative capacity and cellular demise (Elliott *et al.*, 1999b; Ge *et al.*, 1999; Ge *et al.*, 2000a; Sakamuro *et al.*, 1996). To investigate this phenomenon in more detail, we used a set of

recombinant adenoviral vectors that efficiently deliver Bin1 to human cells. One vector was constitutive and used the cytomegalovirus (CMV) early promoter to drive Bin1 expression (Ad-Bin1). A second vector was inducible and allowed Bin1 expression to be controlled by coexpression of Cre recombinase. In this vector, Bin1 cDNA was inserted downstream of a CMV promoter and a stuffer cassette flanked by loxP sites (Ad-MABin1). A matched control virus was also constructed for each system in which a loss-of-function Bin1 gene was expressed (see below). Recombinant viruses were generated in human 293 cells by standard methods. DNA sequencing from amplified viral DNA confirmed the expected structure of each transgene (data not shown). For control experiments, we also prepared adenoviruses expressing LacZ (Ad-LacZ) or Cre recombinase (Ad-cre) in 293 cells. The host cell line used for infection was HepG2 hepatoma cells, a functionally null cell which lacks endogenous Bin1 expression and is susceptible to Bin1-mediated growth suppression (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996).

Infections employing Ad-lacZ indicated that a multiplicity of infection (m.o.i.) of 50-100 was required to infect >90% of HepG2 cells exposed to virus *in vitro* (data not shown). HepG2 cells infected with Ad-Bin1 at 10, 50, or 100 m.o.i. exhibited increasing levels of Bin1 expression as documented by Western analysis of cell extracts prepared and analyzed 48 hr after infection (see Fig. 1A). Bin1 was detectable within 12 hr of infection and reached a maximum by 48 hr (see Fig. 1B). When infected under similar conditions, the level of ectopic Bin1 in IMR90 diploid fibroblasts used in control experiments was comparable to that seen in HepG2 cells (see Fig. 1C). Indirect cell immunofluorescence experiments indicated that the high level of expression driven by Ad-Bin1 was correlated with localization of Bin1 throughout the cell. To document Bin1 expression from the Cre-inducible vector Ad-MABin1, we infected HepG2 cell lines that stably expressed Cre recombinase (HepG2/cre) or that contained only vector sequences (HepG2/CMV). Bin1 was detected in HepG2/cre cells infected with 50 m.o.i. of Ad-MABin1 (see Fig. 1D). In contrast, Bin1 was not detected after infection of HepG2/CMV cells, where the stuffer upstream of Bin1 could not be removed, nor was it detected in either cell line infected with control viruses that lacked an insert (Ad-

vect). A constitutive adenoviral vector was also constructed for a loss-of-function mutant of Bin1, termed Bin1ΔBAR-C, that lacks aa 125-206 within the N-terminal BAR domain which is crucial for antineoplastic activity (Elliott *et al.*, 1999b). Extracts derived from cells infected with Ad-Bin1ΔBAR-C exhibited a polypeptide with the expected apparent mobility of ~50 kD (see Fig. 1E). A second smaller polypeptide was also detected at lower levels relative to the Bin1ΔBAR-C protein on Western blots. The appearance of this polypeptide did not affect the loss-of-function of Bin1ΔBAR-C, which was employed as a negative control for cell death induction (see below). In summary, these experiments validated the adenoviral vectors for constitutive or inducible Bin1 expression in human cells.

HepG2 cells expressing Bin1 displayed striking morphological changes. Cells assumed a rounded, shrunken morphology and exhibited deformations of the plasma membrane before completely detaching from substratum (see Fig. 2A). The changes seen were consistent with induction of PCD like that observed previously (Ge *et al.*, 1999; Ge *et al.*, 2000a). Cells infected with Ad-lacZ at similar or higher m.o.i. did not display similar morphologies. No signs of cellular demise were noted following infections of normal human diploid IMR90 fibroblasts with Ad-Bin1 (see Fig. 2A). IMR90 cells showed some enlargement but flow cytometry indicated no signs of cell cycle arrest (data not shown), consistent with previous observations in rodent embyro fibroblasts, Rat1 fibroblasts, or mouse C2C12 myoblasts (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1998; unpublished observations). Thus, Bin1 was not grossly toxic to cells, consistent with previous observations (Ge *et al.*, 1999; Ge *et al.*, 2000a; Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1998). Induction of the cre-inducible virus Ad-MABin1 produced identical effects. In HepG2/CMV cells, the control virus Ad-vect had little morphological effect whereas uninduced Ad-MABin1 had a slight effect on cell shape (see Fig. 2B). The effect of the latter virus might reflect low leaky expression seen in other cell types infected by Ad-MABin1 (Ge *et al.*, 1999). However, the uninduced luciferase vector Ad-MA19 produced similar effects and we observed no increase in detached cells using either vector (data not shown), arguing for a nonspecific effect of the Ad-MA vector in HepG2 cells. In any case,

there was a profound difference seen in HepG2/cre cells, where Ad-vect had no discernible effect but Ad-MABin1 elicited a dramatic increase in detached cells (see Fig. 2B). Taken together, these results confirmed that the cytopathic effect elicited by the constitutive Ad-Bin1 vector was not due to nonspecific toxicity of the vector system.

To explicitly assess cell viability in infected cultures, cells incubated with various m.o.i. of Ad-Bin1 or Ad-lacZ were harvested 48 hr after infection and the proportion of viable cells was determined by trypan blue exclusion. Cells infected with Ad-lacZ at all m.o.i. were >90% viable whereas cells infected with Ad-Bin1 exhibited a linear relationship between m.o.i. and loss of viability (see Fig. 2C). Similar results were obtained using Ad-MABin1 (data not shown). Thus, the cytopathic phenotype elicited by Bin1 was associated with cell death.

We next examined whether cells induced to die by Bin1 exhibited any differences in endocytosis, because brain-specific splice isoforms of Bin1 that localize to the cytosol in differentiated neurons have been implicated in this process, like the related adaptor protein amphiphysin (David *et al.*, 1996; Ramjaun *et al.*, 1997; Wigge *et al.*, 1997). Ubiquitous splice isoforms of Bin1 that localize to the nucleus lack brain-specific exons required for interaction with clathrin-coated endocytotic vesicles and AP2 (Ramjaun & McPherson, 1998; P. deCamilli, pers. comm.). However, while this structural difference suggested strongly that endocytotic roles were probably brain-specific, we wished to explicitly rule out a role for endocytosis in cell death induction by the nonneuronal Bin1 isoform used in these experiments. Fluid-phase or receptor-mediated endocytosis was monitored by comparing the rate of uptake of horseradish peroxidase or fluorescein-conjugated transferrin, respectively (Barbieri *et al.*, 1998; Benmerah *et al.*, 1998). No differences were apparent in the level of uptake of either reagent in cells infected with Ad-Bin1 or Ad-LacZ (data not shown). These observations supported the expectation that the endocytotic function of the Bin1 gene is regulated by alternate splicing and that splice isoforms that localize to the nucleus do not have these roles (Kadlec &

Pendergast, 1997; Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a; Wechsler-Reya *et al.*, 1998). We concluded that the ability of Bin1 to induce cell death was unrelated to effects on endocytosis.

To begin to define the basis for cell death, we analyzed HepG2 cells by flow cytometry following terminal transferase-catalyzed dUTP labeling of nicked DNA ends (TUNEL assay). Cells were infected with increasing m.o.i. of adenoviral vector and harvested for propidium iodide staining, TUNEL assay, and flow cytometry 48 hours later. A dose-dependent increase in the proportion of TUNEL-positive cells was observed after Ad-Bin1 infection (see Fig. 2D). TUNEL positivity was detected in cells in all phases of the cell cycle as indicating by propidium iodide staining. An increase in cells with sub-G1 phase DNA occurred in the TUNEL-positive population with similar kinetics. A time-course experiment confirmed observations that the appearance of TUNEL-positive cells and the accumulation of cells with sub-G1 phase DNA began 24-36 hr after infection, corresponding to a time approximately 12-24 hr after Bin1 expression (see Fig. 2E). This experiment also highlighted the finding that apoptotic cells (as defined by TUNEL positivity) emerged from all phases of the cell cycle. Taken together, these results suggested that Bin1 induced a programmed cell death (PCD) process. Similar experiments performed with the inducible vector confirmed these observations and confirmed that they were dependent on Bin1 expression rather than a nonspecific effect of the adenoviral vector (data not shown). The features of DNA degradation seen in flow cytometry experiments paralleled the kinetics of morphological features of apoptosis and loss of viability. In multiple trials using each vector system, no reproducible effects of Bin1 on the distribution of cells in the cell cycle were noted, consistent with the observation that TUNEL-positive cells emerged from all phases. Taken together, the results suggested that Bin1 engaged a PCD process that could be initiated at any point in the cell cycle, similar to c-Myc and other oncogenes (Evan *et al.*, 1992).

BAR domain is crucial for Bin1 to induce cell death. The Bin1 BAR domain is crucial to inhibit transformation of rodent fibroblasts by c-Myc and to suppress the proliferation of HepG2 cells (Elliott *et al.*, 1999b). To determine whether this domain was also crucial for death

activity, we infected cells with Ad-Bin1ΔBAR-C, which expresses the loss-of-function mutant Bin1ΔBAR-C (Elliott *et al.*, 1999b). Ad-Bin1ΔBAR-C did not elicit the cell detachment and cytopathic phenotype produced by Ad-Bin1 (see Fig. 3A). Similarly, flow cytometric analysis of cells harvested 48 hr after viral infection showed that Ad-Bin1ΔBAR-C did not elicit DNA degradation, even at elevated multiplicities of infection (see Fig. 3B). These observations provided additional evidence that the cytotoxic effects of Bin1 in HepG2 cells were not due to some nonspecific cause, since overexpression of a loss-of-function mutation that eliminated tumor suppressor properties also abolished PCD activity. We concluded that Bin1 engaged a PCD process that could explain the basis for its tumor suppressor properties.

p53 and Rb are dispensable for PCD by Bin1. p53 has a central role in many types of PCD but it is mutated in many cancers. The retinoblastoma protein (Rb) has been reported to be antiapoptotic in many systems but in some cases it also has proapoptotic roles (Bowen *et al.*, 1998; Day *et al.*, 1997). The central importance of p53 and Rb in PCD control prompted us to determine whether these genes were needed for Bin1 action. HepG2 cells have wild-type p53 and Rb genes, so we examined the effect of Ad-Bin1 on another tumor cell line, SAOS-2 osteosarcoma, which has homozygous deletions in both genes. SAOS-2 cells were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 virus and expression was confirmed by ß-galactosidase staining or Western blotting, respectively (data not shown). Parallel dishes of cells were examined for the appearance of morphological features of apoptosis or harvested and processed for TUNEL labeling and flow cytometry. Similar to its effects on HepG2, Ad-Bin1 caused cell rounding, plasma membrane deformations, and substratum detachment (see Fig. 4A). Flow cytometry showed an increase in the number of TUNEL-positive cells with sub-G1 phase DNA following Ad-Bin1 infection. Positive cells emerged from all phases of the cell cycle and kinetics were similar to those observed in HepG2 cells (see Fig. 4B). These results were consistent with evidence that p53 is dispensable for PCD by c-Myc in epithelial cells (Sakamuro *et al.*, 1995; Trudel *et al.*, 1997) and with findings in breast cancer and melanoma cell lines that p53 status was not correlated with Bin1 susceptibility (Ge *et al.*, 1999; Ge *et al.*, 2000a). Consistent with

the SAOS-2 susceptibility, the results of extensive RNAse protection and Western analyses of HepG2 cells expressing Bin1 did not reveal any differences in the expression of a variety of cell cycle and apoptosis regulators, including p53, Rb family members, cell cycle-dependent kinase inhibitors (p16INK4, p14ARF, p21WAF1, p27KIP1, or p57KIP2), or Bcl-2 family genes (Bcl-2, Bcl-X_L, Mcl-1, Bik, Bax, or Bak). We concluded that Bin1 acted independently of the p53 and Rb pathways.

Bin1 does not activate caspases. The type of PCD process elicited by Bin1 was further characterized by examination of nuclear phenotypes associated with caspase activation. HepG2 cells infected with Ad-Bin1 but not Ad-LacZ displayed signs of nuclear deformation and chromatin margination at the nuclear periphery and at focal sites in the nucleoplasm (see Fig. 5A). However, cells maintained considerable genomic DNA integrity and there was no evidence of nuclear lamina breakdown. This phenotype was different from that seen in classical or type I apoptosis, which is associated with a distinct nuclear condensation phenotype, and suggestive of type II apoptosis in which cytosolic features predominate. The contrast in nuclear morphology was highlighted by comparison to the nuclear phenotype elicited by the protein kinase inhibitor staurosporine, which induces type I apoptosis (see Fig. 5A, right panel). Transient transfection of HepG2 cells with Bin1 plasmid vectors elicited the same nuclear phenotype as Ad-Bin1, ruling out a nonspecific adenovirus vector artifact (data not shown). To determine whether Bin1 caused nucleosomal DNA cleavage ("DNA laddering"), genomic DNA was isolated from cells infected with adenoviral vectors or treated with staurosporine and fractionated by agarose gel electrophoresis. Genomic DNA degradation was observed in cells infected by Ad-Bin1 but not by Ad-lacZ (see Fig. 5B, left panel). This result was consistent with the ability of Bin1 to induce positive TUNEL reaction. However, Bin1 did not induce nucleosomal DNA cleavage, even though this feature could be induced in HepG2 cells by staurosporine (see Fig. 5B, right panel).

The caspase-3-activated nuclease DFF/CAD is primarily responsible for nucleosomal DNA degradation and chromatin collapse which is a characteristic of classical or type I apoptosis (Enari *et*

al., 1998; Liu *et al.*, 1997; Woo *et al.*, 1998). The absence of these features suggested that Bin1 did not activate caspase-3. To confirm this likelihood, caspase-3-like activities were assayed in extracts from Ad-Bin1-infected cells by measuring cleavage of the fluorescent substrate Ac-DEVD-pNA (see Fig. 5C). Cleavage of Ac-DEVD-pNA by caspase-3-like activity was monitored by absorbance at 405 nM measured at various times after addition to extracts. Exogenous recombinant caspase-3 was used as a positive control for the assay. As expected, staurosporine induced caspase-3-like activity in cell extracts. This induction confirmed that HepG2 cells expressed pro-caspase-3-like enzymes that were competent for activation by apoptotic stimuli, in support of evidence of a predominant role for caspase-3 in classical apoptotic responses in HepG2 cells (Suzuki *et al.*, 1998). In contrast, no significant cleavage of Ac-DEVD-pNA occurred in extracts prepared from cells infected with Ad-Bin1 or Ad-LacZ. In Western blotting experiments, we also did not detect proteolytic cleavage of either pro-caspase-3 or the caspase-3 substrate PARP (data not shown).

One possibility was that Bin1 activated a caspase other than caspase-3. To assess this possibility, we tested whether Bin1-induced cell death was suppressed by ZVAD.fmk, a broad spectrum inhibitor of caspases. In these experiments, the relative number of cells displaying sub-G1 phase DNA by flow cytometry was used as a measurement of PCD. As expected, ZVAD.fmk significantly blocked staurosporine-induced cell death. In contrast, ZVAD.fmk did not affect PCD by Bin1 even when added at high concentrations (see Fig. 5D). Taken together, these results argued that the death process activated by Bin1 was caspase-independent.

To confirm this conclusion and gain additional insight into this death process we examined cells by electron microscopy. These experiments revealed cytosolic features consistent with a programmatic death process such as type II apoptosis (see Fig. 6). Ad-lacZ-infected cells showed no signs of cytopathology. In contrast, staurosporine-treated cells were shrunken and exhibited cytosolic vacuolation, chromatin condensation, and nuclear degeneration (plasma membrane blebs were also observed but were not so dramatic in this cell system). Ad-Bin1-infected cells were similarly

shrunken and heavily vacuolated. Margination of chromatin at the nuclear periphery was evident. However, there was no nuclear degeneration and limited chromatin condensation by comparison to staurosporine-treated cells. Cell nuclei were shrunken relative to Ad-LacZ-infected cells but not so severely as staurosporine-treated cells. Strikingly, the nuclear lamina in Ad-Bin1-infected cells remained essentially intact. This feature supported the lack of caspase involvement because lamins are subjected to caspase-mediated proteolysis during PCD (Lazebnik *et al.*, 1993; Rao *et al.*, 1996). In addition, lamin cleavage is separable from chromatin collapse (Lazebnik *et al.*, 1995) but is important for complete nuclear degeneration (Rao *et al.*, 1996), so the more limited nuclear degeneration in Ad-Bin1-infected cells was also consistent with a caspase-independent process. We observed no 'exploded' cells or flocculent densities in organelles or the cytosol that would signal necrosis. Trials in which ZVAD.fmk was added confirmed that the PCD phenotype induced by Bin1 was not affected by caspase inhibition. Addition of 100 μM ZVAD.fmk reduced cell volume and induced the appearance of focal densities in the nucleus, but these features were a nonspecific artifact of ZVAD.fmk, because the same features were also apparent in Ad-LacZ-infected cells. Interestingly, ZVAD.fmk did not block the Bin1 phenotype, in which cytosolic vacuolization predominated. In contrast, ZVAD.fmk dramatically affected the phenotype of staurosporine-treated cells, reversing nuclear degradation to a large extent. We concluded that Bin1 engaged a PCD process that was caspase-independent yet associated with limited chromatin degradation and cytosolic features of PCD.

Cell death by Bin1 is not blocked by Bcl-2 or Fas pathway inhibition. To further delineate the death process activated by Bin1 we investigated links to two classical PCD regulatory pathways. Bcl-2 proteins inhibit caspase activation through their ability to influence mitochondrial physiology whereas death receptors directly activate caspases (Ashkenazi & Dixit, 1998; Thompson & Vander Heiden, 1999). HepG2 cell lines overexpressing Bcl-2 or a dominant inhibitor of FADD, which blocks Fas signals (Muzio *et al.*, 1996), were tested for response to Ad-Bin1. One would predict the response would be unaffected by Bcl-2 or Fas pathway disruption if Bin1 acted in a caspase-independent manner. Cells overexpressing Bcl-2 were resistant to staurosporine-induced

apoptosis relative to vector control cells (data not shown). In contrast, there was no difference in the susceptibility of cells overexpressing Bcl-2 or the FADD dominant inhibitor to death induction by Bin1 (see Fig. 7A). On a different line of work, we had observed that recombinant Bin1 baculoviruses increased the kinetics of cell death in Sf9 cells and that Bcl-2 could not inhibit this effect (see Fig. 7B), despite the fact that Bcl-2 inhibits baculovirus-induced cell death in this system (Alnemri *et al.*, 1992). In support of these results, we also obtained a set of negative results from experiments aimed at determining whether Bin1 caused cytochrome c release or altered mitochondrial membrane potential (data not shown). In summary, we concluded that Bin1 acted via non-classical mechanisms that were independent of the Bcl-2 and Fas pathways, the chief regulators of caspases in cells.

SV40 T antigen inhibits induction of cell death by Bin1. In previous work, we found that Bin1 inhibited Ras co-transformation of primary rodent fibroblasts by c-Myc and adenovirus E1A but not by SV40 large T antigen (Elliott *et al.*, 1999b; Sakamuro *et al.*, 1996). Since Bin1 activated a PCD process in malignantly transformed human cells we hypothesized that T antigen might suppress these effects. This hypothesis was not invalidated by the finding that p53 and Rb were dispensable for Bin1-induced death, because T antigen has additional role(s) in immortalization and human cell transformation beyond inactivation of these tumor suppressors (Conzen & Cole, 1995; Hahn *et al.*, 1999; Powell *et al.*, 1999). The effects of T antigen were examined using WI-38 diploid fibroblasts and a WI-38 derivative that expresses T antigen (WI-38/T cells). Like HepG2, WI-38 cells are functionally null for Bin1, in this case due to a missplicing event which causes loss-of-function identical to that which occurs in Bin1 in melanoma (Ge *et al.*, 1999). Western analysis confirmed RT-PCR results (Wechsler-Reya *et al.*, 1997b) establishing missplicing of brain-specific exon 12A in WI-38 cells and also in the WI-38/T derivative. The 12A isoform exhibited reduced mobility on SDS gels relative to wild-type Bin1 (see Fig. 8A, top panel) and was also recognized by a monoclonal antibody that is specific for exon 12A-encoded residues (Ge *et al.*, 1999) (see Fig. 8A, bottom panel).

T antigen blocked susceptibility to cell death by Bin1 (see Figs. 8B and 8C). Loss of viability occurred only if Ad-MABin1 was induced by coinfection with Ad-cre virus. Death correlated with the appearance of rounded, detached cells similar to the cytopathic seen in HepG2 and other malignant cell lines (Ge *et al.*, 1999; Ge *et al.*, 2000a). This response confirmed that the missplicing and inactivation of Bin1 in WI-38 was functionally meaningful. Similar results were obtained using derivatives of HepG2 that were engineered to express T antigen gave (data not shown). To confirm that endogenous misspliced isoform in WI-38 was truly a loss-of-function alteration, we infected cells with an adenoviral vector expressing this isoform (Ad-MABin1-10+12A) (Ge *et al.*, 1999). No loss of viability or cell detachment was observed. We concluded that SV40 T antigen suppressed PCD by Bin1.

A serine protease implicated in PCD by c-Myc inhibits DNA degradation by Bin1. The serine protease inhibitor AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride) has been reported to inhibit c-Myc-induced death in Rat1 fibroblasts (Kagaya *et al.*, 1997). This report also indicated that AEBSF does not block apoptosis induced by Fas activation, the cytotoxic T cell granule protein granzyme B, or a variety of cytotoxic drugs, nor did other kinds of serine protease inhibitors affect death by c-Myc. Thus, AEBSF was a relatively specific inhibitor of a feature of the death process(es) induced by c-Myc. We reasoned that if there was any overlap in the mechanisms used by Bin1 and c-Myc, then AEBSF might affect the death process activated by Bin1. To test this notion, HepG2 cells were infected with Ad-LacZ or Ad-Bin1, or treated with staurosporine, and then left untreated or treated with AEBSF or its inactive analog AEBSA (4-(2-aminoethyl)benzenesulfonamide). Flow cytometry was used to monitor the appearance of sub-G1 phase DNA as a measurement of PCD. In contrast to ZVAD.fmk, AEBSF suppressed Bin1-induced DNA degradation to the background levels seen in cells infected with Ad-LacZ (see Figure 9). This suppression reflected inhibition of a serine protease(s), rather than some nonspecific effect, because the inactive compound AEBSA was ineffective. Consistent with previous findings that AEBSF is not a general inhibitor of apoptosis (Kagaya *et al.*, 1997), under the same conditions where AEBSF

suppressed the action of Bin1 it did not suppress DNA degradation induced by staurosporine (the effects of which were actually accentuated by AEBSF treatment). Thus, AEBSF specifically inhibited DNA degradation by Bin1. We concluded Bin1 acted in part through activation of a serine protease, similar to c-Myc, in support of the conclusion that Bin1 and c-Myc shared certain PCD mechanisms.

Discussion

Bin1 activates a PCD process in malignant cells. This study defines a function for the BAR family adaptor protein Bin1 in a caspase-independent PCD process that limits neoplastic transformation. Bin1 function is complex and regulated by alternate splicing. Splice isoforms that interact with c-Myc have tumor suppressor properties and are missing or inactivated in breast carcinoma, metastatic prostate cancer, and malignant melanoma (Ge *et al.*, 1999; Ge *et al.*, 2000a; Ge *et al.*, 2000b; Sakamuro *et al.*, 1996). The results of this study suggest that the antineoplastic effects of these Bin1 isoforms in malignant cells are a consequence of their ability to activate PCD. Caspase-independent death processes where cytosolic features predominate without accompanying classical nuclear phenotypes have been termed type II apoptosis. The mechanisms underlying this type of PCD are poorly understood. Two other cancer suppression genes, Pml and CD47, have been reported to activate suicide programs with similar features. Pml is a nuclear dot-associated protein that is disrupted in acute promyelocytic leukemia. Expression of wild-type Pml in malignant cells engages a death program with features similar to that engaged by Bin1 (Quignon *et al.*, 1998). CD47 is a receptor for the antiangiogenic extracellular matrix protein thrombospondin. CD47 ligation engages a death program on chronic lymphocytic leukemia cells (Mateo *et al.*, 1999). It is unclear whether these programs share any common aspect. Nevertheless, such caspase-independent processes may be important because they are evolutionarily ancient: "classic" apoptotic regulators such as CED-4, Bak, and Bax elicit death in yeast, with vacuolation phenotypes similar to those seen in metazoan cells, despite the fact that yeast lacks caspases (Ink *et al.*, 1997; James *et al.*, 1997; Jurgensmeier *et al.*,

1997; Xiang *et al.*, 1996). Such programs may be integrated yet distinct from apoptosis, which apparently evolved later in metazoan cells.

Independence from mitochondrial processes and caspases. We found that PCD by Bin1 was not subject to suppression by Bcl-2 or to inhibition of the Fas pathway and that cellular demise was not associated with mitochondrial alterations. These observations were internally consistent given that the death process was caspase-independent. However, they were unexpected given that Bin1 interacts with c-Myc, which activates classical apoptosis by eliciting cytochrome c release and subsequent caspase activation (Juin *et al.*, 1999).

Two important issues impact consideration of this apparent conundrum. First, it seems likely that c-Myc acts in a complex manner to induce death. Cytochrome c release has been shown to be an important component of the process activated by c-Myc, but this event is apparently insufficient (Juin *et al.*, 1999). In addition, as noted above, careful investigations have shown that neither Bcl-2 nor caspase inhibitors block the death commitment decision induced by c-Myc, but merely the phenotype and kinetics of the execution step (McCarthy *et al.*, 1997). Interestingly, caspase-independent suicides that occur retain the cytosolic but not the nuclear features of classical apoptosis (McCarthy *et al.*, 1997), not unlike the death phenotype induced by Bin1. A distinction between the mechanisms which affect death kinetics and those which affect death commitment decisions are crucial, because in slow growing tumors where c-Myc is overexpressed (such as many carcinomas), the effects of Bcl-2 or other antiapoptotic signals may be insufficient to provide adequate escape from the death penalty that is associated with c-Myc overexpression. If this is the case, there would be a selection for loss of caspase-independent processes that impact execution and that are separate from the mitochondrial and death receptor axes.

A second issue is the possibility that caspase-independent steps are required for apoptotic outcomes by c-Myc or other proapoptotic stimuli. A precedent for this concern is raised by studies of

the tumor suppressor Pml, which activates caspase-independent death in tumor cells but which is also necessary for apoptosis induced by a wide variety of death stimuli (Quignon *et al.*, 1998; Wang *et al.*, 1998). It is interesting that the death processes used by c-Myc or Bin1 are each susceptible to inhibition at some level by AEBSF, a serine protease inhibitor which does not generally affect apoptosis (Kagaya *et al.*, 1997). Serine death proteases and caspases appear to act in various cells in different but integrated hierarchies (Kagaya *et al.*, 1997; Wright *et al.*, 1997), so the fact caspases are differentially activated by c-Myc and Bin1 supports the notion of more than one death signal emerging from c-Myc. Preliminary results from our laboratory using dominant inhibitory and antisense approaches support the possibility that Bin1 may be necessary for apoptosis by c-Myc (unpublished observations). However, due to the unusual caspase-independent nature of the Bin1 death phenotype in malignant cells, we are addressing this question further using cells targeted for Bin1 gene deletion. In summary, despite the key role of apoptotic escape in malignant development, it is notable that caspases and other apoptosome components appear to be inactivated in cancer cells less frequently than caspase-independent functions such as Pml or Bin1 (Ge *et al.*, 1999; Ge *et al.*, 2000a; Ge *et al.*, 2000b; Mu *et al.*, 1994; Sakamuro *et al.*, 1996; Zhang *et al.*, 2000). Our findings suggest that Bin1 participates in some caspase-independent process which can influence cell death commitment in malignant cells.

Susceptibility to inhibition by SV40 T antigen. We found that the death process activated by Bin1 was susceptible to suppression by SV40 large T antigen. The ability of this tumor virus protein to block the death process supported the notion that its inactivation is relevant to cancer development. This result was also consistent with earlier findings that transformation of rodent fibroblasts by T antigen is not subject to suppression by Bin1 (Sakamuro *et al.*, 1996). This observation does not conflict with the finding that Bin1 acts independently of p53 and Rb, because there is evidence that T antigen acts beyond inactivating p53 and Rb in immortalization and human cell transformation (Conzen & Cole, 1995; Hahn *et al.*, 1999; Powell *et al.*, 1999). For example, T antigen cooperates with activated Ras and the telomerase catalytic subunit TERT to cause malignant

transformation of human cells, and one can not complement the effects of T antigen by coexpression of the human papilloma virus E6 and E7 proteins (Hahn *et al.*, 1999), which inactivate p53 and Rb, respectively. In this context, it is interesting to note that before the discovery of TERT the rare successes achieved in immortalizing human cells were achieved usually with T antigen or SV40 DNA. Studies of how T antigen can immortalize rodent embryo fibroblasts support the notion of a specialized function beyond p53 and Rb inactivation (Conzen & Cole, 1995; Powell *et al.*, 1999). Interestingly, Bin1 is inactivated by missplicing in diploid WI-38 cells in the same manner as in malignant melanoma (Ge *et al.*, 1999) and this event makes them susceptible to killing by Bin1., unlike IMR diploid fibroblasts. Thus, Bin1 inactivation may be relevant to WI-38 biology, perhaps affecting some process also targeted by T antigen.

Bin1 function. Bin1 has two functions that are varied by alternate splicing. The most significant alterations in Bin1 occur in brain. Brain isoforms localize to clathrin-coated vesicles and promote synaptic vesicle endocytosis, like amphiphysin, by recruiting enzymes that modify lipids and alter membrane structure (Wigge & McMahon, 1998). In contrast, the ubiquitous Bin1 isoforms lack sequences needed for targeting to clathrin-coated vesicles (Ramjaun & McPherson, 1998) and instead exhibit nuclear localization (Elliott *et al.*, 1999b; Wechsler-Reya *et al.*, 1997a; Wechsler-Reya *et al.*, 1998). The c-Myc-interacting isoform analyzed in this study did not affect endocytosis under conditions where it activated cell death. Preliminary examination of Bin1-10, a closely related c-Myc-interacting isoform, suggest it has similar properties (unpublished observations). In contrast, isoforms that include brain exon 12A did not induce death, consistent with previous results (Ge *et al.*, 1999). Thus, we believe that the death induction function of Bin1 is a unique feature of isoforms that can localize to the nucleus and interact with c-Myc. Bin1 isoforms form heterodimers in the brain (Wigge *et al.*, 1997). Therefore, it is tempting to speculate that the endocytosis connection in neurons reflects a specialized link in those cells between survival and the achievement of a synaptically active state, which would be associated with neurotransmitter release and hence endocytotic membrane trafficking.

From a functional standpoint, although ubiquitous Bin1 isoforms are not amphiphysin-like, the high conservation between amphiphysin and the ubiquitous Bin1 isoforms nonetheless suggest a functional connection to membrane dynamics at some level (perhaps related to internal vesicle dynamics). The BAR domain comprises the major part of this conservation. This domain is crucial for death activity but what it does is currently obscure. A membrane connection is intriguing in light of emerging interest in possible connections between autophagy and apoptosis (Thompson & Vander Heiden, 1999). However, we have not observed any death inhibitory effects of 3-methyladenine, a classical inhibitor of autophagy (unpublished observations). Nevertheless, there is certainly a precedent for a nucleocytosolic adaptor protein that has dual functions in transcriptional regulation and membrane dynamics. CtBP/BARS is an adaptor protein initially identified through its ability to interact with and inhibit the tumorigenic activity of adenovirus E1A (Schaeper *et al.*, 1995). CtBP/BARS binds to cell cycle and transcriptional regulatory complexes (Meloni *et al.*, 1999; Sewalt *et al.*, 1999; Sollerbrant *et al.*, 1996) but it also controls membrane vesiculation in the Golgi complex (Weigert *et al.*, 1999). Parallels between CtBP and Bin1 are intriguing given their connections to E1A and c-Myc, which are biologically distant cousins. A complex nature for Bin1, like that exhibited by CtBP/BARS, would not be out of step with the complex and still largely obscure nature of c-Myc, which has a highly integrated cell regulatory function.

We did not detect any changes in the expression of several target genes linked to apoptosis by c-Myc, including ornithine decarboxylase, CDC25A, or Fas ligand (Galaktionov *et al.*, 1996; Hueber *et al.*, 1997; Packham & Cleveland, 1994), but no target gene identified to date has been assigned an unambiguous role in apoptosis (Dang, 1999; Evan & Littlewood, 1998). In transient assays, Bin1 can suppress c-Myc transactivation and repress transcription when tethered to DNA (Elliott *et al.*, 1999a). It is unclear whether these activities are physiologically germane; if they are epiphenomenon of transient assays, then Bin1 may have a signaling role that is unaffiliated with transactivation. The latter possibility can be entertained since not all biological actions of c-Myc can be ascribed strictly to

gene regulation (Gusse *et al.*, 1989; Lemaitre *et al.*, 1995; Li *et al.*, 1994; Prendergast & Cole, 1989; Yang *et al.*, 1991) and because Bin1 more resembles a signaling protein than a transcription factor. Based on existing data, we have suggested that PCD by c-Myc involves distinct "priming" and "triggering" steps, the former of which is associated with gene regulation but the latter of which is not (Prendergast, 1999). Further investigations are required to unravel the physiological relationship between Bin1 and the proapoptotic and transcriptional properties of c-Myc, if any.

Bin1, cell death, and cancer. Cancer is characterized by dysfunctional adhesion and cell survival signaling. Our findings suggest that loss of Bin1 may eliminate one mechanism which can limits the consequences of inappropriate activation of c-Myc or other oncogenes. Suicide mechanisms are progressively eliminated during neoplastic progression (Williams, 1991), and in invasive breast cancers and metastatic prostate cancers where Bin1 losses occur frequently (Ge *et al.*, 2000a; Ge *et al.*, 2000b) there is strong evidence that loss of cell suicide capacity corresponds with malignant conversion (Kyprianou *et al.*, 1991; Kyprianou *et al.*, 1990; McDonnell *et al.*, 1992). However, malignant conversion is associated generally with altered adhesive capabilities that facilitate invasion and metastasis. Resistance to anoikis (adhesion deprival-induced cell suicide) is likely to be a crucial feature of the pathophysiology of epithelial malignancy. Interestingly, cell death by many oncogenes including c-Myc is suppressed by integrin signaling (Crouch *et al.*, 1996), and Bin1 was identified to interact with certain α-integrins (Wixler *et al.*, 1999). The interaction between Abl kinase and Bin1 in cells (Kadlec & Pendergast, 1997) would be consistent with an integrin connection, given evidence that Abl can become activated at focal adhesions where integrins are localized (Lewis *et al.*, 1996; Taagepera *et al.*, 1998). Therefore, further investigation of the links between Bin1-dependent cell death and integrin signaling may provide insights into the significance of Bin1 losses in cancer as well as to mechanisms that govern cell death induction by c-Myc and other oncogenes.

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Figure Legends

Figure 1. Recombinant Bin1 adenoviruses. Western analysis of whole cell lysates prepared from cells infected with the adenoviral vectors indicated was performed. The anti-Bin1 monoclonal antibodies 99D were used as the primary antibodies in panels A, B, and D; antibodies 99D plus 99I were used in panel C (Wechsler-Reya *et al.*, 1997a). **(A.)** Expression from the constitutive vector Ad-Bin1. HepG2 cells lacking endogenous Bin1 were incubated with viruses at the multiplicity of infection (m.o.i.) indicated and extracts were prepared 48 hr later. **(B.)** Ad-Bin1 time course. Extracts were prepared from HepG2 cells harvested at the times indicated. **(C.)** Comparative levels of expression in IMR90 diploid fibroblasts or HepG2 cells. Extracts were prepared 48 hr after infection at the indicated m.o.i. IMR90 express endogenous Bin1 (Sakamuro *et al.*, 1996) but at a level that is undetectable on this exposure of the blot, which illustrates similar steady-state levels of Bin1 in IMR90 or HepG2 cells infected with the same amount of Ad-Bin1 **(D.)** Cre-inducible expression from Ad-MABin1. HepG2 cell lines stably expressing P1 bacteriophage Cre site-specific recombinase (HepG2/cre) or containing only vector sequences (HepG2/CMV) were incubated with the virus indicated (m.o.i. = 100) and extracts were prepared 48 hr later. The transgene in Ad-MABin1 is located downstream of a loxP site-flanked stuffer sequence such that expression occurs only after Cre-induced recombination (see Materials and Methods). Ad-vect is a control virus that contain no transgene. **(E.)** Expression of the loss-of-function deletion mutant Bin1ΔBAR-C. Western analysis was performed using extracts isolated from cells infected with 100 m.o.i. Ad-Bin1 or Ad-Bin1ΔBAR-C, which lacks anti-transforming and tumor suppressor properties (Elliott *et al.*, 1999b).

Figure 2. Bin1 induces demise of malignant cells. (A.) Morphology of HepG2 hepatoma cells or IMR90 diploid fibroblasts following infection with Ad-LacZ or Ad-Bin1. Cells were incubated with 100 m.o.i. virus indicated and photographed 48 hr later using phase-contrast optics (magnification = 100x). **(B.)** Morphology of cells infected with inducible Ad-MABin1 virus. HepG2 cells stably expressing Cre recombinase or vector only were incubated with 100 m.o.i. virus

indicated and processed as above. **(C.)** Viable cell count. Cells were infected with the viruses indicated, as in Figure 1, and harvested by trypsinization 48 hours later. The proportion of viable cells in the population was determined by trypan blue exclusion. **(D.)** Cell death is associated with DNA degradation and occurs throughout the cell cycle. Cells were incubated with the indicated m.o.i. of Ad-LacZ or Ad-Bin1 and processed 48 hr later for TUNEL reaction, propidium iodide staining, and flow cytometry. In each graph, the X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity. Time courses indicated a correlation between the kinetics of the cytopathic effect and DNA degradation. **(E.)** Cell death elicited by Bin1 occurs throughout the cell cycle. Cells were incubated with 50 m.o.i. Ad-LacZ or Ad-Bin1 and processed after the period indicated for TUNEL reaction, propidium iodide staining, and flow cytometry. In each graph, the X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity.

Figure 3. BAR deletion abolishes cell death by Bin1. (A.) Lack of cytopathic effect. HepG2 cells were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 and photographed 48 hr later (magnification = 100x). **(B.)** Flow cytometry. Cells were infected with the m.o.i. virus indicated and harvested and processed 48 hr after infection for flow cytometry.

Figure 4. p53 and Rb are dispensable for programmed cell death by Bin1. SAOS-2 osteosarcoma cells, which have homozygous deletions of p53 and Rb, were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1. **(A.)** Morphology. Cells were photographed 48 hr after infection (magnification = 100x). **(B.)** Flow cytometry. Cells were harvested 48 hr after infection and processed for TUNEL assay and propidium iodide staining. Representative results from analysis by flow cytometry are shown. The X-axis corresponds to relative PI staining and the Y-axis to the log of the FITC signal reflecting relative TUNEL positivity.

Figure 5. Lack of caspase activation or requirement. **(A.)** Hoescht nuclear stain. HepG2 cells seeded on cover slips were infected with 100 m.o.i. Ad-LacZ or Ad-Bin1 and 48 hr later fixed and stained with Hoescht dye, mounted, and examined by immunofluorescence microscopy. A separate culture was treated with 0.5 μM staurosporine as a positive control for apoptotic morphology. Cells were photographed at 400x magnification. **(B.)** Nucleosomal DNA degradation. HepG2 cells were infected with viruses or treated with staurosporine as above. Hirt DNA (left panel) or total genomic DNA (right panel) was prepared and analyzed by agarose gel electrophoresis. **(C.)** Caspase-3 assay. HepG2 cells were infected with m.o.i. 100 Ad-LacZ or Ad-Bin1 or treated with 0.5 μM staurosporine and harvested 36 hr later. Extracts were prepared and assayed for the presence of caspase-3-like activity using the colormetric substrate Ac-DEVD-pNA. Reactions were monitored for production of cleavage product at 405 nm at the times indicated. Recombinant caspase-3 was used as a positive control. **(D.)** Caspase inhibition does not block Bin1-induced cell death. HepG2 cells were infected with 100 m.o.i. Ad-Bin1 or treated with 0.5 μM staurosporine in the presence or absence of the caspase inhibitor ZVAD.fmk at the concentration indicated. Cells were harvested 36 hr later and processed for flow cytometry. The graph shows the relative proportion of the cell population undergoing cell death, using the appearance of sub-G1 phase DNA as an indicator.

Figure 6. Death phenotype. HepG2 cells were infected with m.o.i. 200 Ad-LacZ or Ad-Bin1 or treated with 0.5 μM staurosporine as above, stained with osmium tetroxide, and processed for electron microscopy using standard methods. Where indicated, cells were treated with 100 μM ZVAD.fmk. The bars under each panel represent 2 μm.

Figure 7. PCD by Bin1 is not inhibited by Bcl-2 or Fas pathway inhibition. **(A.)** Bcl-2 or Fas pathway inhibition does not block Bin1-induced cell death. HepG2 cells overexpressing Bcl-2 or a dominant inhibitory mutant of the Fas-interacting adaptor protein FADD (Muzio *et al.*, 1996) were infected with 100 m.o.i. Ad-vect or Ad-MABin1 plus 100 m.o.i. Ad-cre and viability was measured by trypan blue exclusion 48 hr later. *Inset*, Western analysis showing expression of Bcl-2

and FADD dominant negative (FADD DN) proteins relative to vector controls for each expression construct. **(B.)** Increased kinetics of insect cell death elicited by recombinant Bin1 baculovirus are unaffected by Bcl-2. Sf9 cells were infected as described (Alnemri *et al.*, 1992; Elliott *et al.*, 1999b) and the proportion of viable cells in the culture were determined at various times post-infection. *Inset*, Western analysis of cell extracts processed at 24 hr after infection demonstrating expression of Bin1 and Bcl-2.

Fig. 8. SV40 T antigen blocks cell death induction by Bin1. (A.) Western analysis demonstrates missplicing of exon 12A in Bin1 in WI-38 diploid fibroblasts. Previous results from RT-PCR experiments demonstrated the presence of exon 12A in Bin1 messages expressed in WI-38 cells (Wechsler-Reya *et al.*, 1997b). *Top panel*, extracts prepared from C2C12 myoblasts (positive control), WI-38 cells, and WI-38 cells transformed by SV40 T antigen (WI-38/T cells; also known as VA13 cells) were analyzed with anti-Bin1 mAb 99D (Wechsler-Reya *et al.*, 1997a). The arrow indicates a slower mobility band in WI-38 and WI-38/T consistent with the presence of aberrantly spliced isoform. *Bottom panel,* extracts prepared from the human melanoma cell line WM793 (positive control), WI-38 cells, and WI-38/T cells were analyzed with anti-12A mAb (Ge *et al.*, 1999). The arrow indicates a polypeptide including exon 12-derived residues in WI-38 and WI-38/T that has identical mobility to the aberrant Bin1-10+12A splice isoform expressed in human melanoma (Ge *et al.*, 1999). **(B.)** WI-38 cells are susceptible to Bin1 PCD, which is suppressed by SV40 T antigen. Cells were infected with 100 m.o.i. adenoviral vector indicated plus 100 m.o.i. Ad-cre and viable cells were determined by trypan blue exclusion 48 hr later. **(C.)** Cytopathic effect of Bin1 in WI-38 cells and its inhibition by SV40 T antigen. Cells were processed as above and photographed 48 hr after viral infection.

Figure 9. Implication of a serine protease(s) in programmed cell death by Bin1. HepG2 cells were infected with 100 m.o.i. Ad-Bin1 or Ad-LacZ or treated with 0.5 μM staurosporine and left untreated or treated additionally with 0.4 mM AEBSF or its inactive analog AEBSA. Cells

were processed for flow cytometry 24 hr later. The graph presents the relative proportion of the cell population exhibiting sub-G1 phase DNA. The mean and standard error of three trials is shown.

FIGURE 1

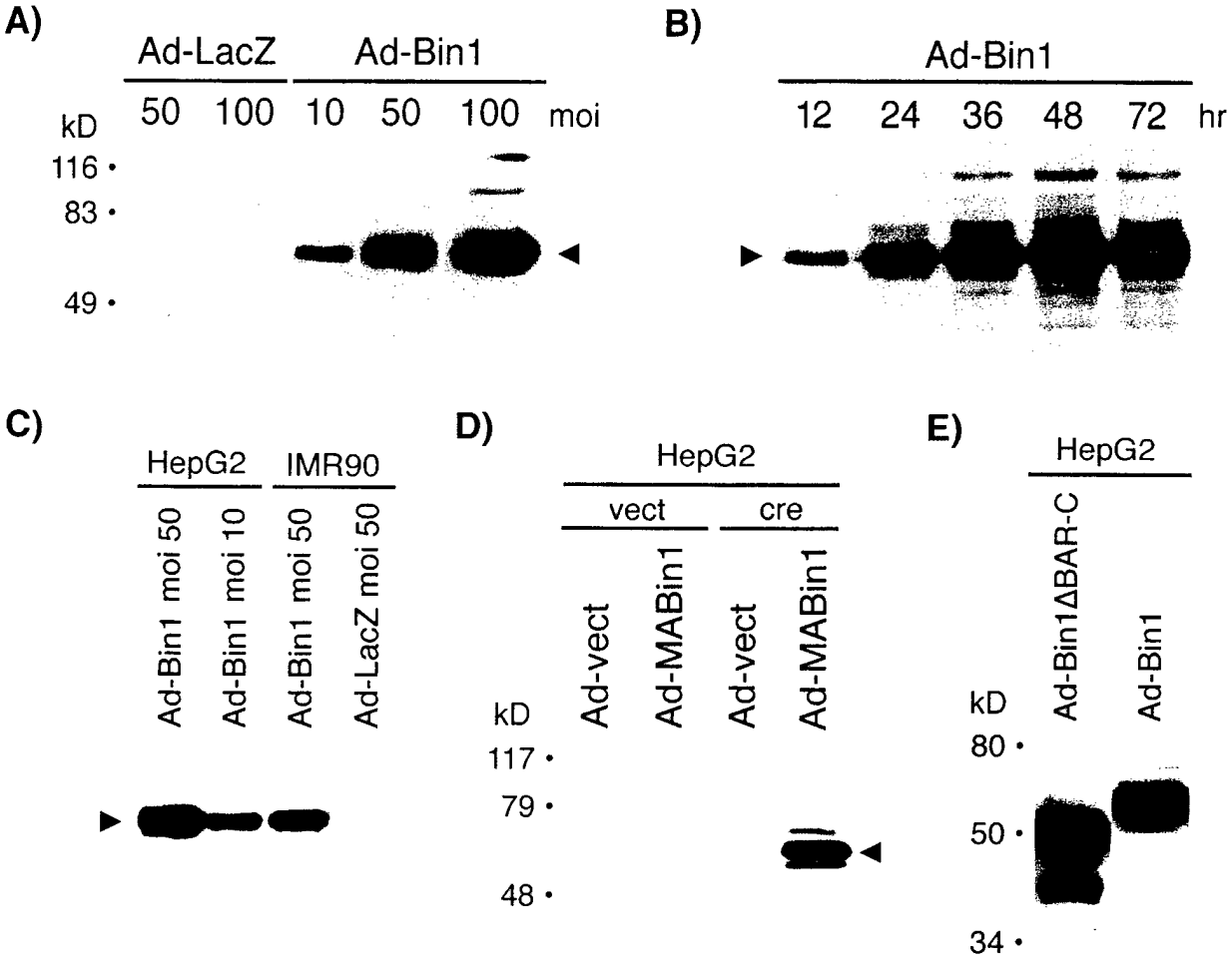

A)
Ad-LacZ
Ad-Bin1
50
100
10
50
100
moi
kD
116
83
49
B)
Ad-Bin1
12
24
36
48
72
hr
C)
HepG2
IMR90
Ad-Bin1 moi 50
Ad-Bin1 moi 10
Ad-Bin1 moi 50
Ad-LacZ moi 50
D)
HepG2
vect
cre
Ad-vect
Ad-MABin1
Ad-vect
Ad-MABin1
kD
117
79
48
E)
HepG2
Ad-Bin1ΔBAR-C
Ad-Bin1
kD
80
50
34

FIGURE 2

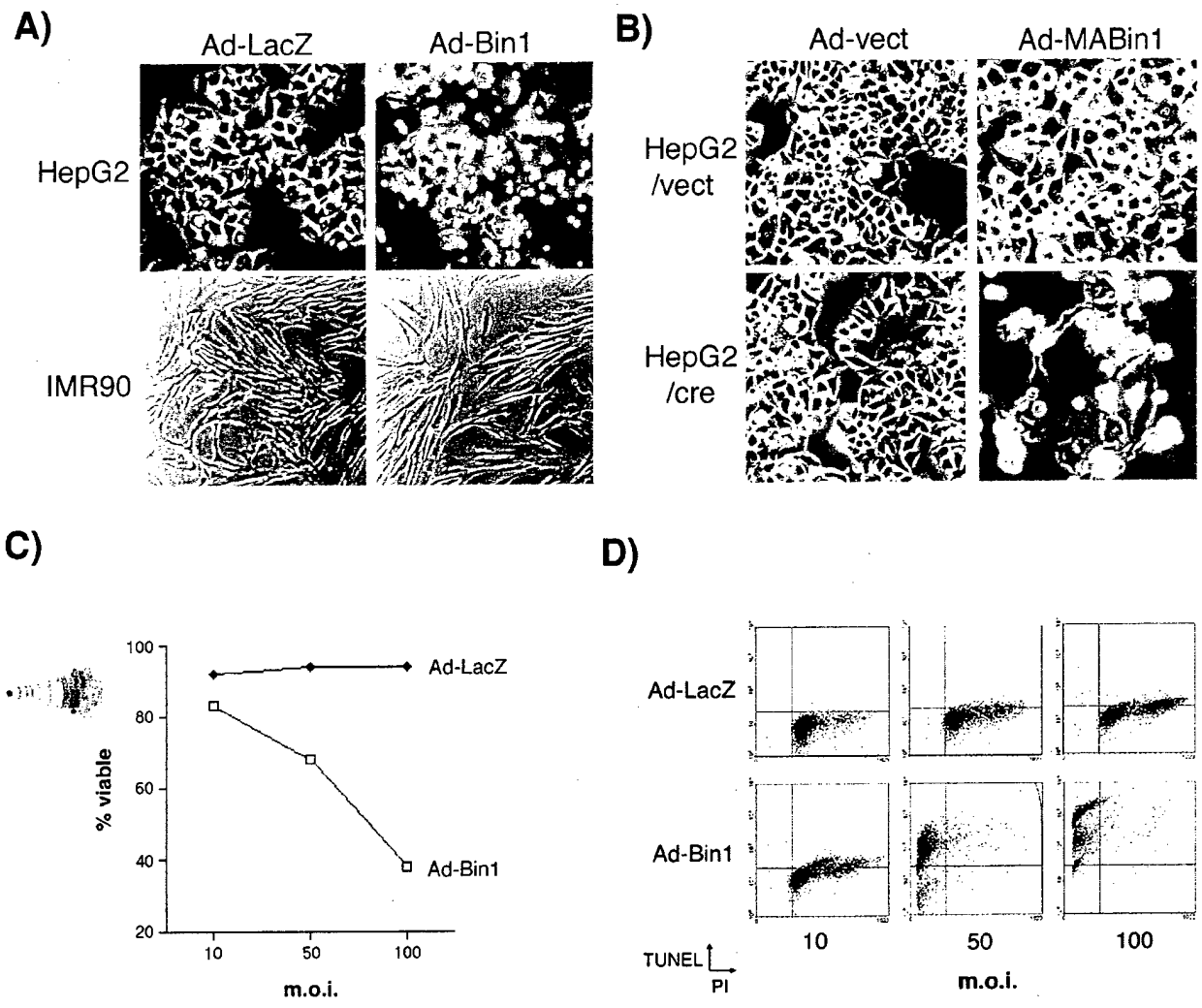

A)
Ad-LacZ
Ad-Bin1
HepG2
IMR90
B)
Ad-vect
Ad-MABin1
HepG2 /vect
HepG2 /cre
C)
100
80
60
40
20
% viable
Ad-LacZ
Ad-Bin1
10
50
100
m.o.i.
D)
Ad-LacZ
Ad-Bin1
TUNEL
PI
10
50
100
m.o.i.

FIGURE 2

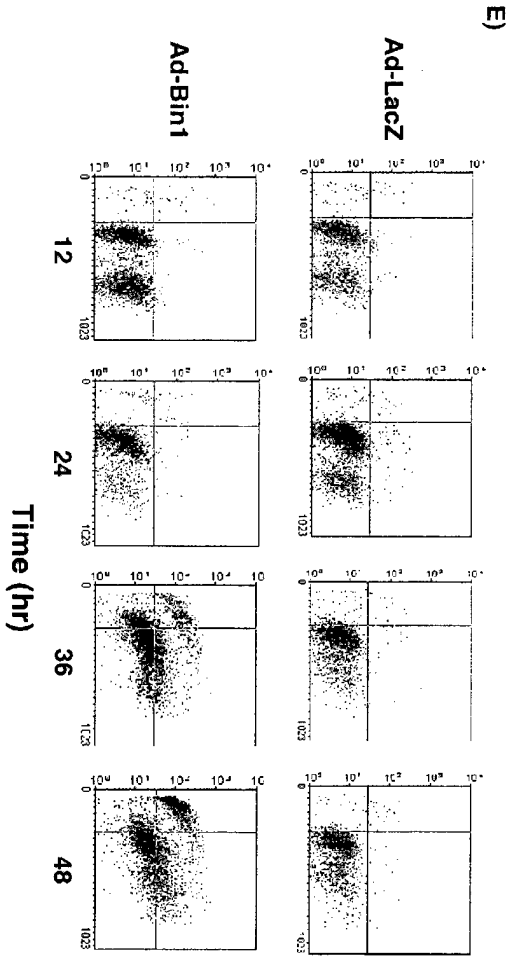


FIGURE 3

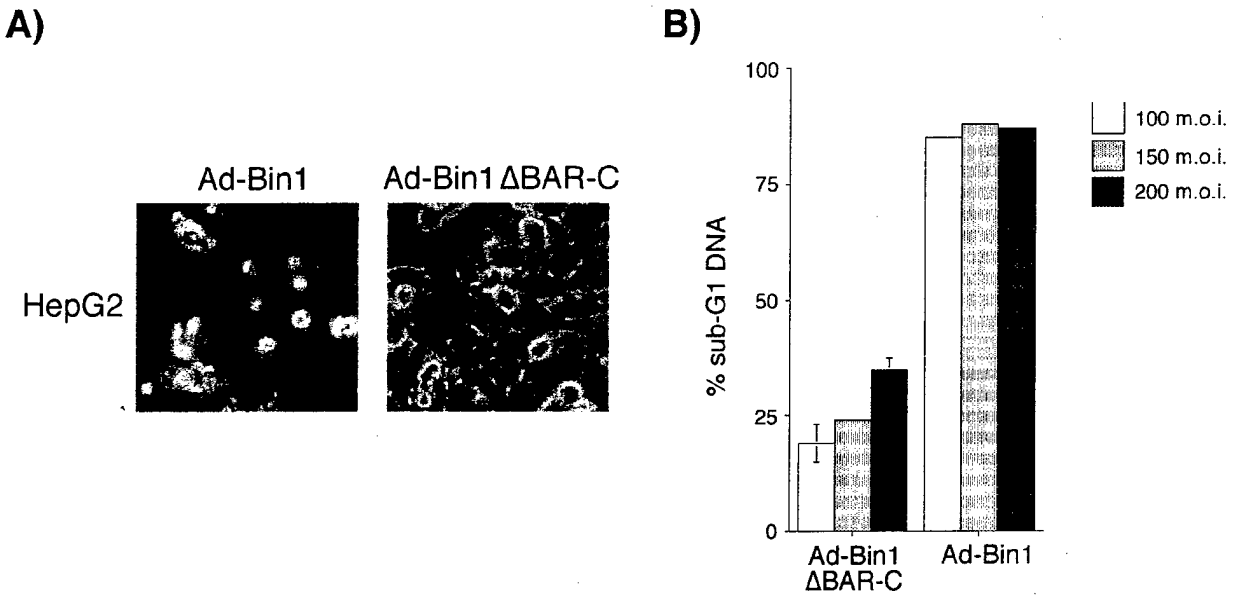

A)
Ad-Bin1
Ad-Bin1 ΔBAR-C
HepG2
B)
% sub-G1 DNA
100
75
50
25
0
Ad-Bin1 ΔBAR-C
Ad-Bin1
100 m.o.i.
150 m.o.i.
200 m.o.i.

FIGURE 4

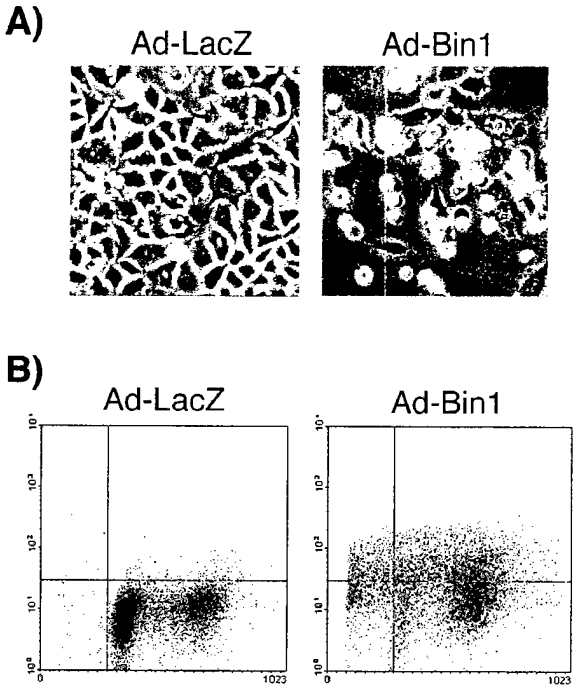


FIGURE 5

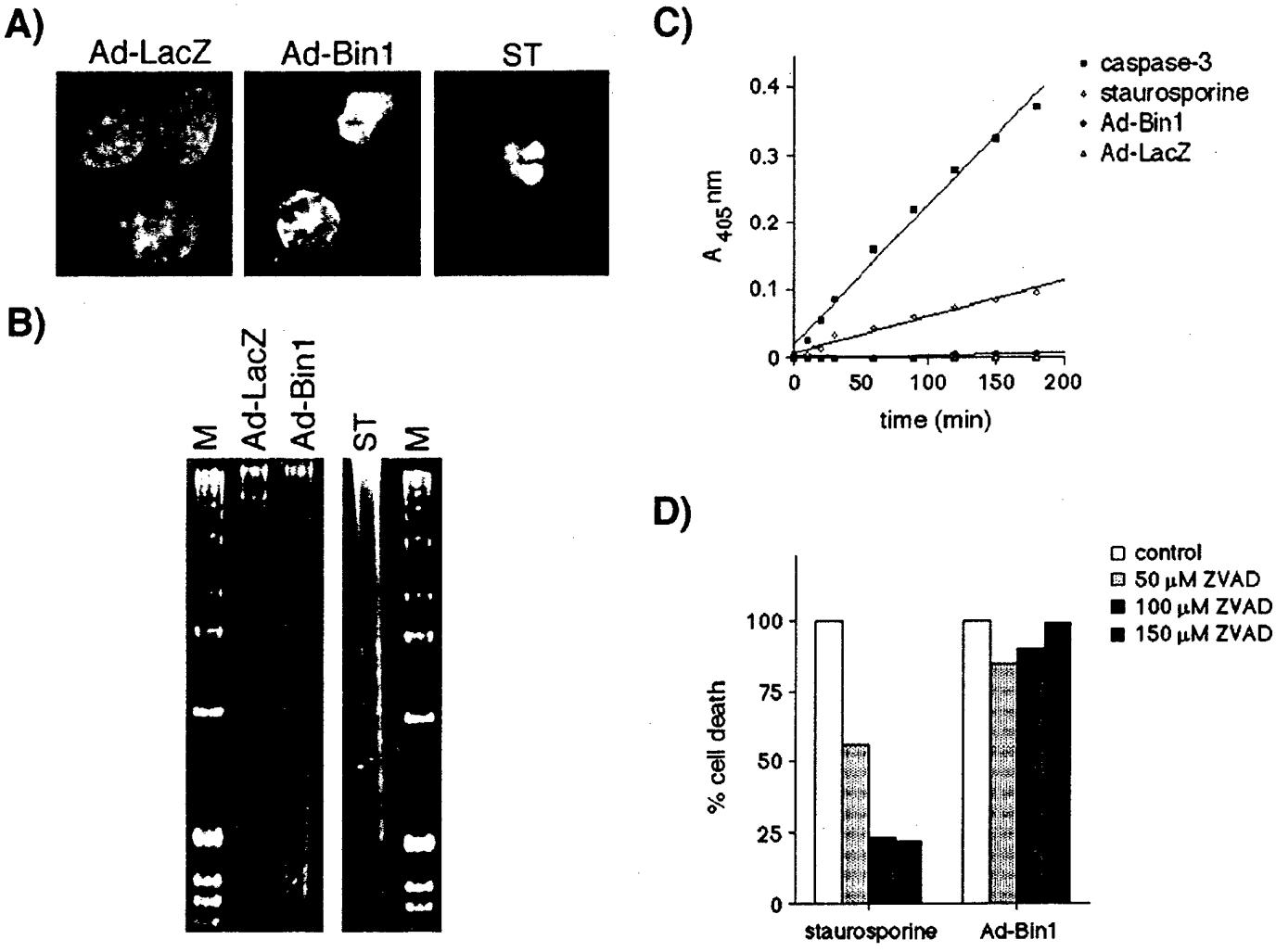

A)
Ad-LacZ
Ad-Bin1
ST
B)
M
Ad-LacZ
Ad-Bin1
ST
M
C)
caspase-3
staurosporine
Ad-Bin1
Ad-LacZ
A_{405} nm
0.4
0.3
0.2
0.1
0
0
50
100
150
200
time (min)
D)
control
50 μM ZVAD
100 μM ZVAD
150 μM ZVAD
% cell death
100
75
50
25
0
staurosporine
Ad-Bin1

FIGURE 6

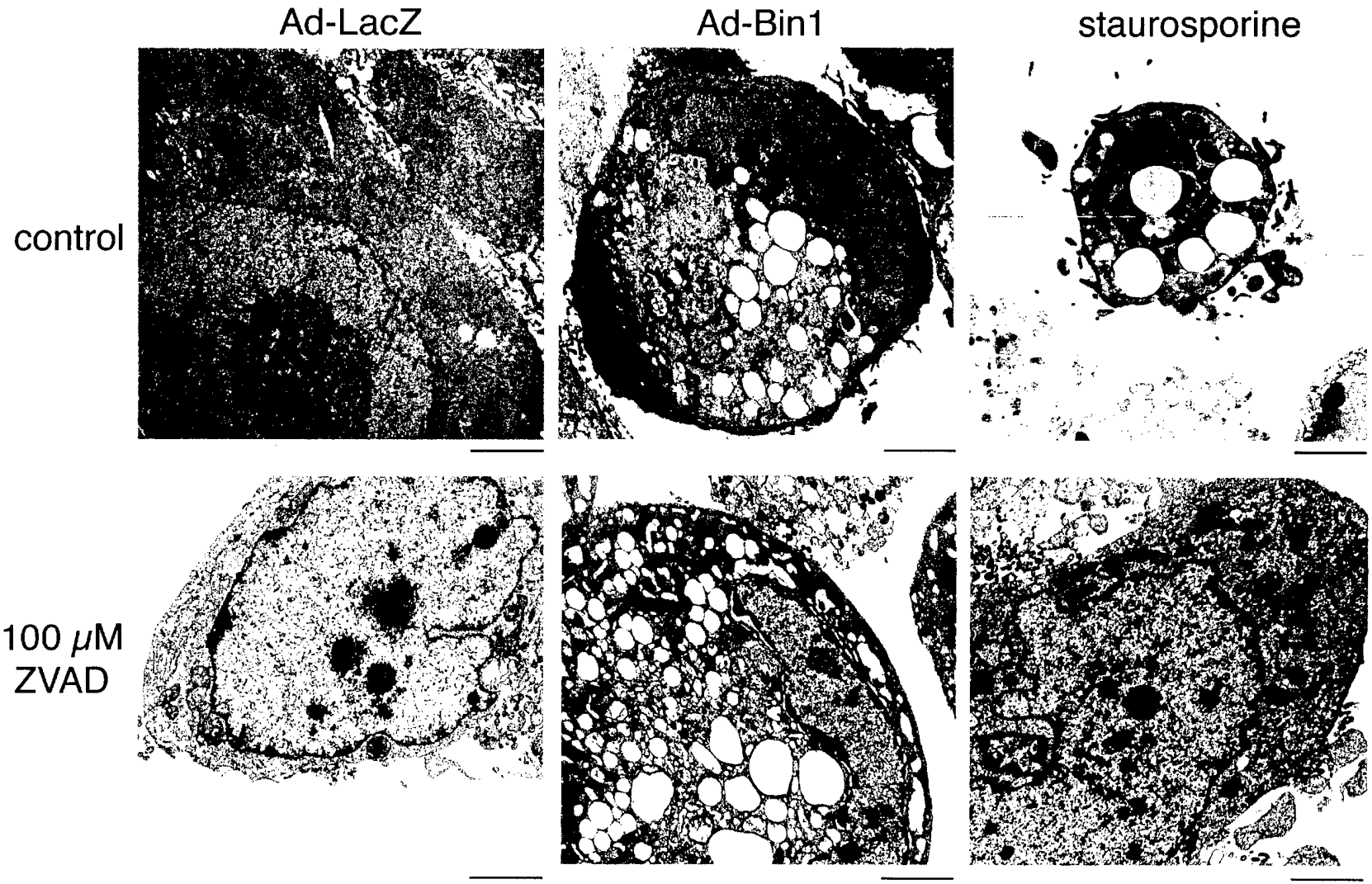


FIGURE 7

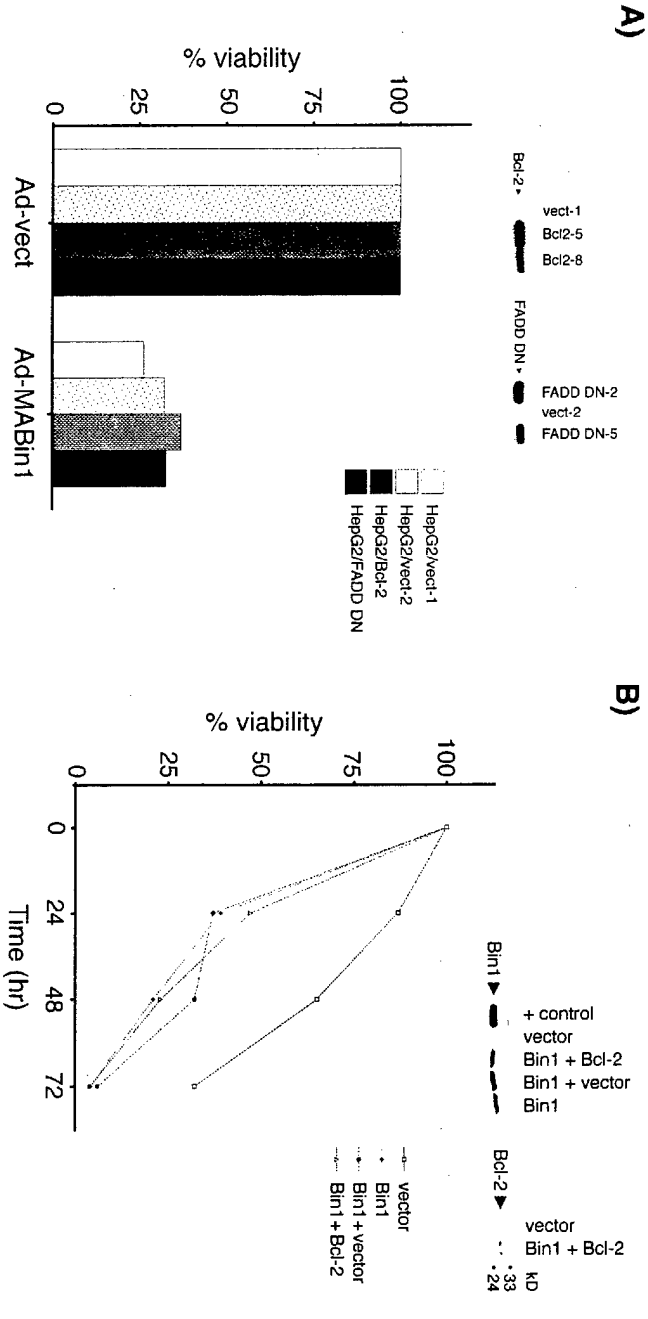

A)
% viability
0
25
50
75
100
Ad-vect
Ad-MABin1
Bcl-2
vect-1
Bcl2-5
Bcl2-8
FADD DN
FADD DN-2
vect-2
FADD DN-5
HepG2/vect-1
HepG2/vect-2
HepG2/Bcl-2
HepG2/FADD DN
B)
% viability
0
25
50
75
100
Time (hr)
0
24
48
72
Bin1
+ control vector
Bin1 + Bcl-2
Bin1 + vector
Bin1
vector
Bin1
Bin1 + vector
Bin1 + Bcl-2
Bcl-2
vector
Bin1 + Bcl-2
kD
33
24

FIGURE 8

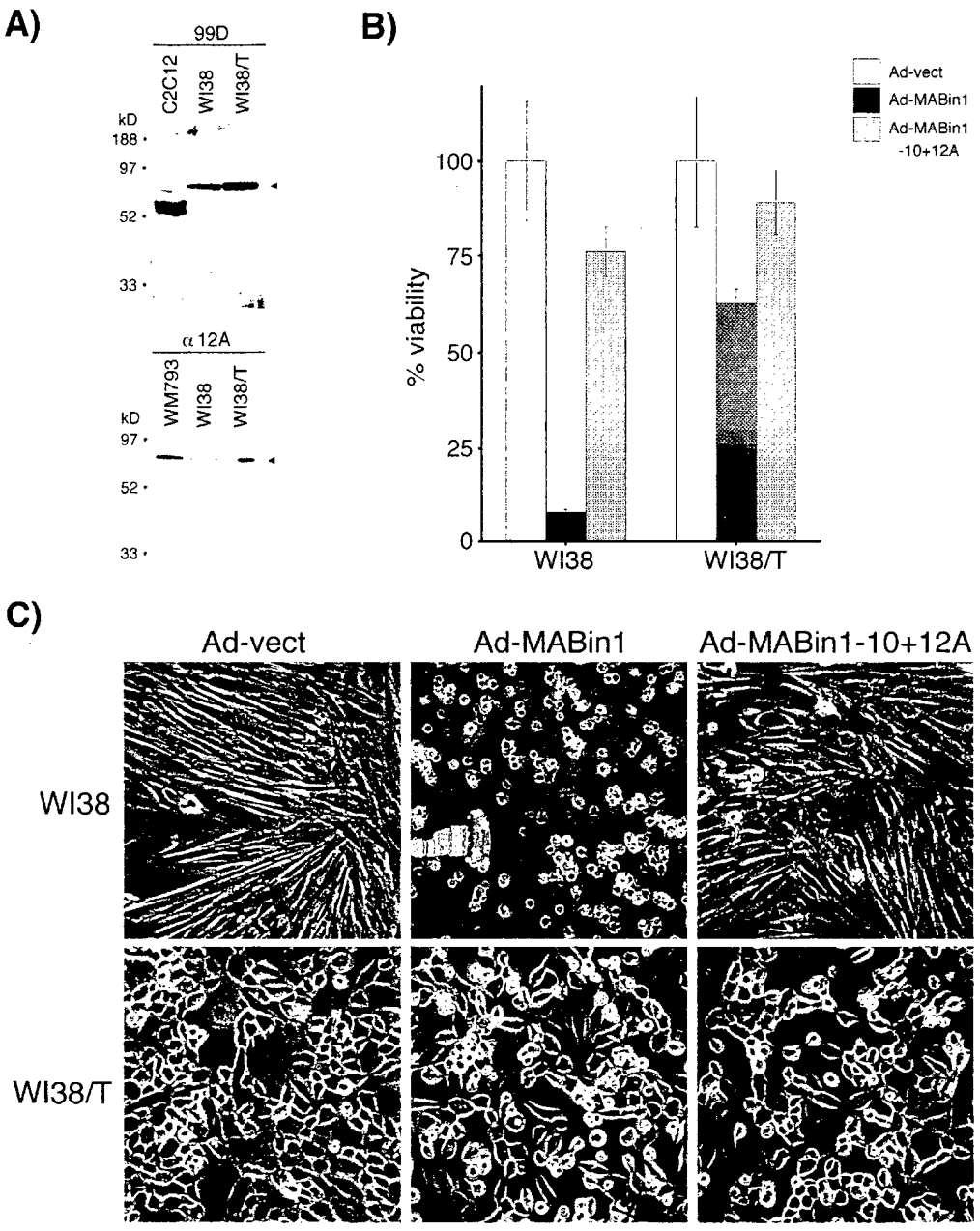
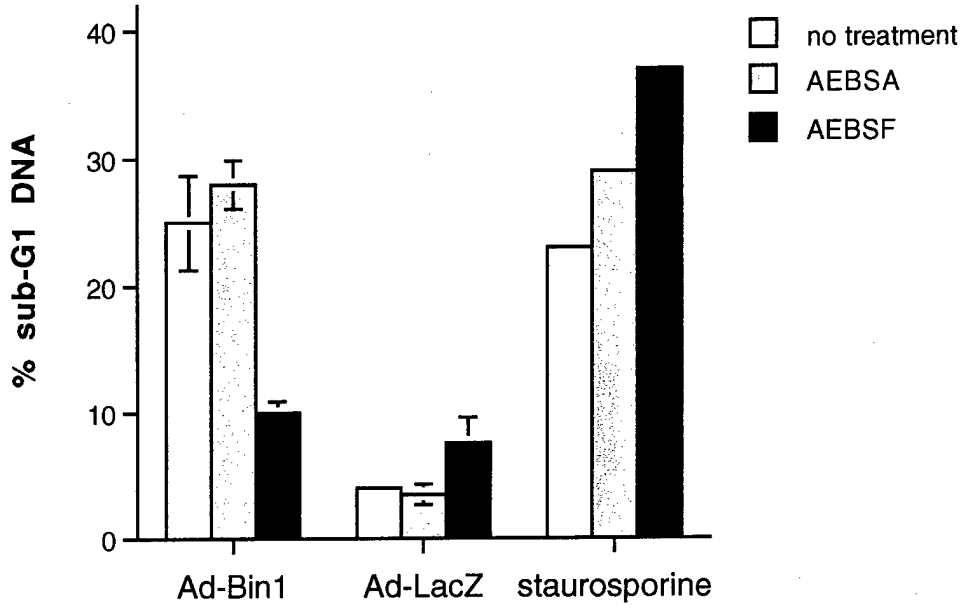

A)
99D
C2C12
WI38
WI38/T
kD
188
97
52
33
α12A
WM793
WI38
WI38/T
kD
97
52
33
B)
Ad-vect
Ad-MABin1
Ad-MABin1 -10+12A
% viability
100
75
50
25
0
WI38
WI38/T
C)
Ad-vect
Ad-MABin1
Ad-MABin1-10+12A
WI38
WI38/T

Fig. 9


% sub-G1 DNA
40
30
20
10
0
Ad-Bin1
Ad-LacZ
staurosporine
no treatment
AEBSA
AEBSF

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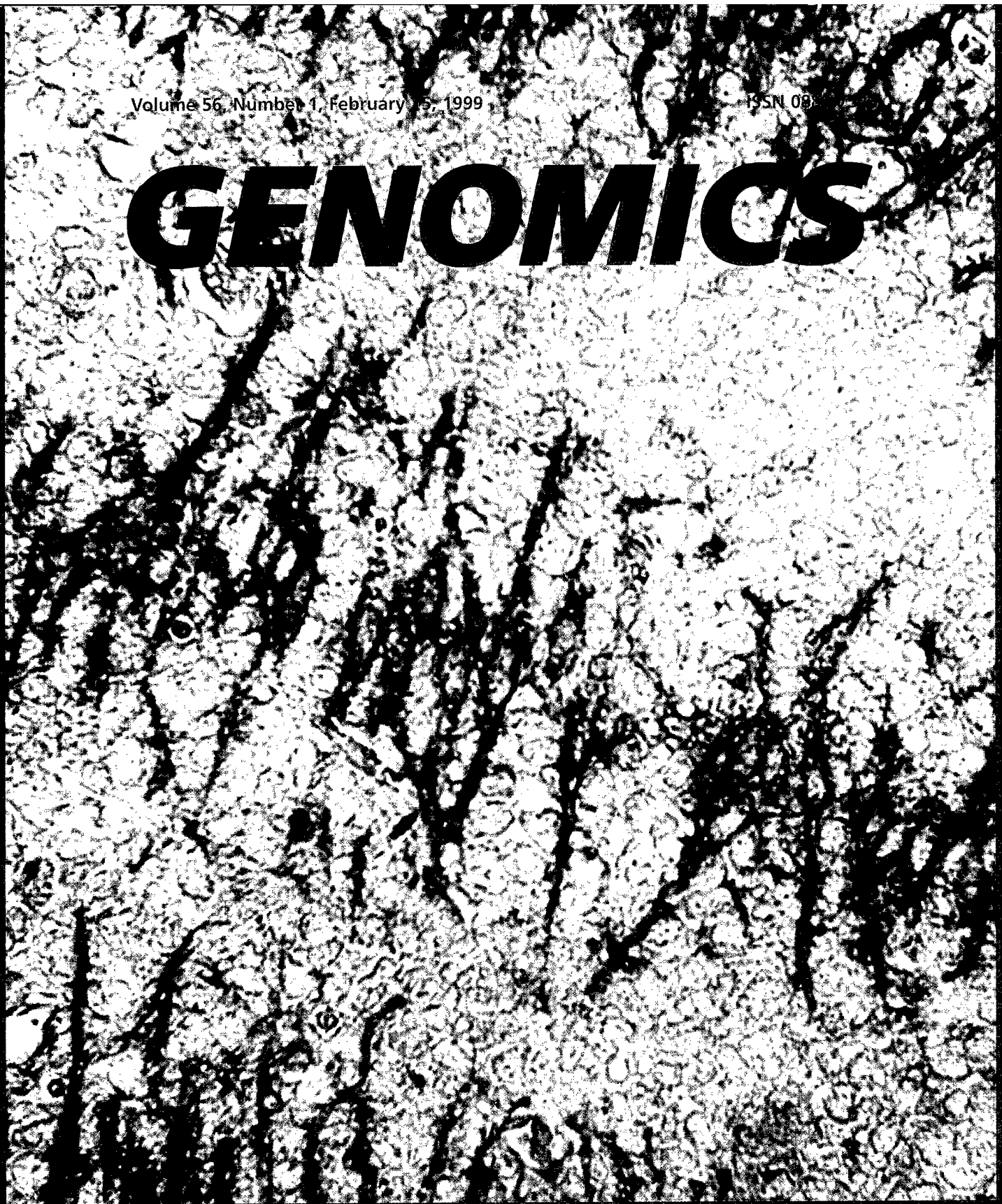
GENOMICS



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The Murine *Bin1* Gene Functions Early in Myogenesis and Defines a New Region of Synteny between Mouse Chromosome 18 and Human Chromosome 2

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We cloned and functionally characterized the murine *Bin1* gene as a first step to investigate its physiological roles in differentiation, apoptosis, and tumorigenesis. The exon–intron organization of the ≥55-kb gene is similar to that of the human gene. Consistent with a role for *Bin1* in apoptosis, the promoter included a functional consensus motif for activation by NF-κB, an important regulator of cell death. A muscle regulatory module defined in the human promoter that includes a consensus recognition site for myoD family proteins was not conserved in the mouse promoter. However, *Bin1* is upregulated in embryonic development by E10.5 in myotomes, the progenitors of skeletal muscle, supporting a role in myogenesis and suggesting that the mouse and human genes may be controlled somewhat differently during development. In C2C12 myoblasts antisense *Bin1* prevents induction of the cell cycle kinase inhibitor p21WAF1, suggesting that it acts at an early time during the muscle differentiation program. Interspecific mouse backcross mapping located the *Bin1* locus between *Mep1b* and *Apc* on chromosome 18. Since the human gene was mapped previously to chromosome 2q14, the location of *Bin1* defines a previously unrecognized region of synteny between human chromosome 2 and mouse chromosome 18.

INTRODUCTION

The identification and functional analysis of tumor suppressor genes are major goals of cancer research. Bin1 is a tumor suppressor in breast and prostate carcinoma that was identified through its ability to interact with the transcriptional regulatory domain of the Myc oncoprotein (Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1997a). Bin1 inhibits the oncogenic and transcriptional properties of Myc (Sakamuro *et al.,* 1996; Elliott *et al.,* in press) but it can also inhibit cell growth by Myc-independent mechanisms (Elliott *et al.,* in press). A necessary role has been defined for Bin1 in differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998). Recent work has identified Abl as a second oncoprotein that interacts with Bin1, through its SH3 domain (Kadlec and Pendergast, 1997) that is dispensible for Myc interaction (Sakamuro *et al.,* 1996). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein that is a paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.,* 1994; Dropcho, 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.,* 1993; Crouzet *et al.,* 1991). Amphiphysin has been implicated in receptor-mediated endocytosis (David *et al.,* 1996; Wigge *et al.,* 1997b), and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin-related protein (Butler *et al.,* 1997; Ramjaun *et al.,* 1997; Tsutsui *et al.,* 1997), have been reported to interact with amphiphysin and to influence endocytosis (Wigge *et al.,* 1997a; Owen *et al.,* 1998). Taken together, the results suggest that Bin1 is a nucleocytoplasmic adaptor that participates in a signaling pathway(s) linking membrane trafficking with gene and cell cycle regulatory events.

The human *BIN1* gene has been cloned and characterized (Wechsler-Reya *et al.,* 1997b). It is ubiquitously expressed and extensively alternately spliced with highest expression in skeletal muscle. *BIN1* is located at chromosome 2q14 (Negorev *et al.,* 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.,* 1996). We cloned the mouse *Bin1* gene (alternate symbol *Amphl*) as a prerequisite to generating homozygous null animals that

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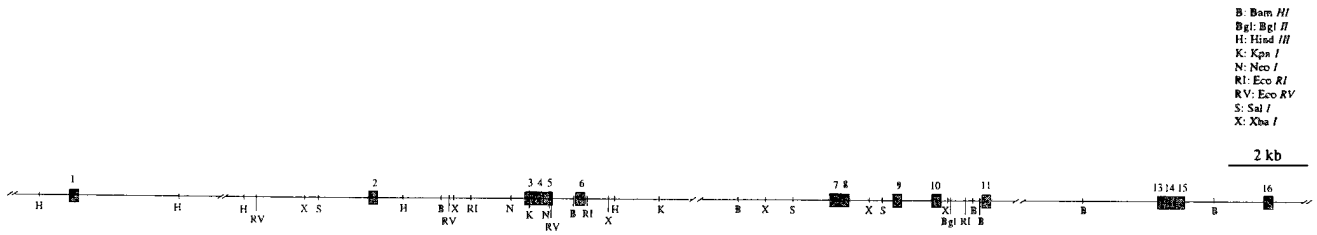

FIG. 1. Physical map of the mouse *Bin1* gene. A restriction map of four contiguous regions of the gene derived from subclones from a single BAC clone is shown. Exons located by Southern analysis with a murine cDNA and limited DNA sequencing were numbered by comparison to the human gene (Wechsler-Reya *et al.,* 1997b).

will allow investigations of its physiological functions. In this study, we defined the exon–intron organization, promoter, and muscle-specific expression of mouse *Bin1*. We also obtained evidence supporting a function for *Bin1* in regulating apoptosis and myogenesis. Finally, we mapped the chromosomal location of the gene to mouse chromosome 18, a locus that defines a new region of synteny with human chromosome 2.

MATERIALS AND METHODS

Gene cloning and characterization. Three clones were isolated from a murine 129/Sv BAC library (Genome Systems) by hybridization with a full-length human cDNA (Sakamuro *et al.,* 1996). Restriction fragments were subcloned into pKS II (−) (Stratagene) and analyzed by extensive restriction mapping and Southern analysis with human Bin1 cDNA probes. The DNA sequences of exon-containing subclones were determined using an automated DNA sequencer. Primer sequences were derived from a full-length murine cDNA, SH3P9 (Sparks *et al.,* 1996), which encodes the ubiquitously expressed splice form of Bin1 lacking exon 10 sequences (Bin1-10) (Wechsler-Reya *et al.,* 1997b,1998). Exon 16 was derived from a mouse ES cell genomic fragment generated by PCR, using primers in exon 15 and 16, because none of BAC clones included this exon. Exon 16 sequences shown in Fig. 2 include sequences from the ES subclone as well as from SH3P9 (Sparks *et al.,* 1996).

DNA sequence analysis. The sequence data were assembled manually with assistance from MacVector and AssembLIGN software. Exons were defined by alignment and comparison to the human *BIN1* gene (Wechsler-Reya *et al.,* 1997b) with additional alignments to Bin1 expressed sequence tags in GenBank. Similarity between the mouse and the human coding regions was computed using the BLASTN and TBLASTN algorithms. Promoter sequences were aligned using CLUSTAL W (1.7) and analyzed with a muscle module detection algorithm (Wasserman and Fickett, 1998) that identifies clustered transcription factor binding sites characteristic of muscle-specific promoters.

Immunohistochemistry. Mouse embryos (10.5 days) were fixed for 24 h in 10% neutral buffered formalin, dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Five micrometer-thick sections were cut and mounted on Snowcoat X-tra Micro slides (Surgipath), air-dried, and heat-fixed for 30 min at 56°C. Slides were deparaffinized in xylene twice for 10 min each and then rehydrated in decreasing percentages of ethanol, starting at 100% and ending in PBS. Endogenous peroxidase was quenched by incubating for 15 min in 1% H_2O_2 in methanol followed by PBS washing. Slides were then placed in a 600-ml beaker in a slide rack containing 500 ml of 10 mM citrate buffer (pH 8.5), covered with plastic wrap, and microwaved for 5-min intervals for a total of 10 min at the highest power setting (Catoretti *et al.,* 1992). After slides were cooled in the citrate buffer for 20 min, antibody staining was performed essentially as described (Sakamuro *et al.,* 1996). Briefly, tissue was blocked with 10% goat serum and incubated for 30 min with a 1:1500 dilution of Bin1 99I monoclonal antibody from ascites (Wechsler-Reya *et al.,* 1997a). The primary antibody was visualized by a 30-min incubation with biotin-conjugated goat anti-mouse antibody, a 30-min incubation with peroxidase-conjugated streptavidin, and a brief treatment with diaminobenzidine. Before mounting, slides were counterstained with an acidified solution of the cytoplasmic dye light green. Stained embryos were photographed using Kodak T64 film on a Leitz microscope at 80× or 400× magnification.

Western analysis. C2C12 cells expressing antisense Bin1 or containing vector only were cultured in growth or differentiation medium and cell extracts were prepared and processed for Western analysis as described (Wechsler-Reya *et al.,* 1998). Blots were probed with rabbit anti-p21 polyclonal antibody C21 (Santa Cruz Biotechnology) or with murine anti-myosin monoclonal antibody MF20 (obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA). HRP-coupled goat anti-mouse or anti-rabbit IgG (BMB) was used with a commercial chemoluminscence kit (Pierce) to develop the blots. Equal protein loading per lane was subsequently confirmed on blots by staining with Ponceau S.

Interspecific mouse backcross mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × *Mus spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). Haplotype analysis of 164 N_2 mice was performed to map the *Bin1* locus; for gene order determination up to 193 mice were typed for some pairs of markers. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins *et al.,* 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, a 1.4-kb *Eco*RI/*Hin*dIII fragment of mouse *Bin1* cDNA, was labeled with [α-^{32}P]dCTP using a random priming kit (Amersham); washing was performed to a final stringency of 1.0 × SSCP, 0.1% SDS at 65°C. The probe detected 13.0-, 10.5-, 8.0-, 5.0-, 4.6-, and 0.5-kb *Bam*HI fragments in C57BL/6J DNA and 10.0-, 8.0-, 6.6-, 5.4-, 4.7-, 2.7-, and 0.9-kb fragments in *Bam*HI-digested *M. spretus* DNA. The presence or absence of the 6.6-, 5.4-, 2.7-, and 0.9-kb *M. spretus*-specific fragments, which cosegregated, was followed in backcross mice. A description of probes and restriction fragment length polymorphisms (RFLPs) for loci linked to *Bin1,* including desmoglein-3 (*Dsg3*), meprin1, β subunit (*Mep1b,*) and the adenomatosis polyposis coli gene (*Apc*) has been reported previously (Gorbea *et al.,* 1993; Ishikawa *et al.,* 1994). Recombination distances and gene orders were determined using Map Manager, version 2.6.5 (Manly, 1993); gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS AND DISCUSSION

Structure of the Murine Bin1 Gene

A physical map of *Bin1* was determined from a single BAC genomic clone that included the entire locus within an ~100-kb insert (see Fig. 1). The relative location of each exon was determined by Southern analysis, and exon and proximal intron sequences were determined by DNA sequencing (see Fig. 2). The mouse

No.	EXON	INTRON
1	CTCACTCGCTCTCCCCGCGCACGCTCCGTCTCCGTCAGTCCCCTGAGCTG TTCTAGTGCGCGGCGTGGAGCCAGGGCTCAGGCTGGTGGAGCGGCCGGGG CTGGAGGCTGGGAGTGCGGCGCGCACGGCCTCCCCGCGCCATTATCCGCG CTCGCTTCGGGCGAGGCCGGCGCCAGGATGGCAGAGATGGGGAGCAAGGG GGTGACGGCGGGGAAGATCGCCAGCAACGTACAGAAGAAGCTGACCCGAG CGCAGGAGAAG	gtgagtgaacggtaaacctgccacacctctcgcccctacccccgggatct ---- > 10 kb ---- ccggcccctggcttctcaaggtgatgttgcccnttntctctgtgcggcag
2	GTCCTGCAGAAACTGGGGAAGGCGGACGAGACGAAGGACGAGCAGTTTGA GCAGTGTGTCCAGAACTTCAATAAGCAGCTG	gtgagtgtttatggaggtgggacagcgtttgctaggtaggagatggtgag ---- ~ 6 kb ---- ttggtgacagggtccccaggacctgaccttgttcttggcttttctggcag
3	ACAGAGGGTACCCGGCTGCAGAAGGATCTTCGGACCTATCTGGCTTCTGT TAAAG	gtagggtacgtctctctgtaaggatttggggctgtcaagctgaggtgggc ---- ~ 150 bp ---- tgtatgagcccgagtggcctggcagggatgaagcaaacaagattgcagag
4	CGATGCACGAAGCCTCCAAGAAGCTGAGTGAGTGTCTTCAGGAGGTGTAT GAGCCCGAGTGGCCTGGCAGGGATGAAGCAAACAAGATTGCAGAG	gtaagcatgggtgggtgccttggttcttcccccaaggccctttttggctt ---- ~ 300 bp ---- tgcagacagtgtggcttagctctacaaacgctcctgtttctatgtttcag
5	AACAATGACCTACTCTGGATGGACTACCACCAGAAGCTGGTGGACCAGGC TCTGCTGACCATGGACACCTACCTAGGCCAGTTCCCTGATATCAAG	gtaagaaacctctgggcccattgtcttgcttgggttggagcttgtggaag ---- ~ 1.3 kb ---- gggatcttggcaccaggccccanngatctctcctcctctctgctccctag
6	TCGCGCATTGCCAAGCGGGGGCGGAAGCTGGTGGACTATGACAGTGCCCG GCACCACTATGAGTCTCTTCAAACCGCCAAAAAGAAGGATGAAGCCAAAA TTGCCAAG	gttcccanctgtgggtggggatggtgctgantccagngccacatanaaca ---- > 10 kb ---- ggtagagttccacacagacgctgacgtaccccactgcctctccatcccag
7	GCAGAAGAGGAGCTCATCAAAGCCCAGAAGGTGTTCGAGGAGATGAACGT GGATCTGCAGGAGGAGCTGCCATCCCTGTGGAACAG	gtaaagtcaggaggggcccaggaacctggcgttcagcctggccctgtgtc ---- ~ 200 bp ---- catcccgtcgcatatggttctcaccatgtcacctcctatctctctggcag
8	CCGTGTAGGTTTCTATGTCAACACGTTCCAGAGCATCGCGGGTCTGGAGG AAAACTTCCATAAAGAGATGAGTAAG	gtaggccaggggactgggctgtgcaaaggatcagtcagaggcagggatg ---- ~ 2 kb ---- tgacgaagatgctcgtccaagcctgcttctcttcactcttcctcctgcag
9	CTCAATCAGAACCTCAATGATGTCCTGGTCAGCCTAGAGAAGCAGCACGG GAGCAACACCTTCACAGTCAAGGCCCAACCCAG	gtaggttagggcagggagggtgaggtcagtggggccctgtggcatgatgg ---- ~ 1.5 kb ---- ttcctagctttcttccaaatgaagcatccacactccaaccatccccacag
10	AAAGAAAAGTAAACTGTTTTCGCGGCTGCGCAGAAAGAAGAACAG	gtaccgccttgagtgcagtgccacgggcctctgggccccgccctgactgc ---- ~ 1.9 kb ---- ctggctctttgttctgttgataccactctcggtctgtccttcttttacag
11	TGACAATGCCCCTGAGAAAGGGAACAAGAGCCCGTCACCTCCTCCAGATG GCTCCCCTGCTGCTACCCCTGAGATCAGAGTGAACCATGAGCCAGAGCCG GCCAGTGGGGCCTCACCCGGGGCTACCATCCCCAAGTCCCCATCTCAG	gtaggcacgactgttatctctatgtcctggttttcttctctttctctttc ---- > 6 kb ---- ggatgcatcctgctctgtatctgatcccttgctggcattttatgttgcag
13	CCAGCAGAGGCCTCCGAGGTGGTGGGTGGAGCCCAGGAGCCAGGGGAGAC AGCAGCCAGTGAAGCAACCTCC	gtaagacggcaggggccgggccctgtttttttcttccctgttgtctgtctg ---- ~ 300 bp gcttttcctacatggccattggtccagctgactcatcccctatccctcag
14	AGCTCTCTTCCGGCTGTGGTGGTGGAGACCTTCTCCGCAACTGTGAATGG GGCGGTGGAGGGCAGCGCTGGGACTGGACGCTTGGACCTGCCCCCGGGAT TCATGTTCAAG	gtgagcgtaggctagccaactctgtagcctttgtctccggtgccttgggg ---- ~ 200 bp tggttaagatgggggaatagccccctganatgccttcttaatttntacag
15	GTTCAAGCCCAGCATGATTACACGGCCACTGACACTGATGAGCTGCAACT CAAAGCTGGCGATGTGGTGTTGGTGATTCCTTTCCAGAACCCAGAGGAGC AG	gtgaacaagggtgtggggaatcccctggctgctgatgcaatggtgggcat ---- ~ 3 kb---- tggtgagtgctgtgtggtgctcctgtgttagccatgctctgttggccccag
16	GATGAAGGCTGGCTCATGGGTGTGAAGGAGAGCGACTGGAATCAGCACAA GGAACTGGAGAAATGCCGCGGCGTCTTCCCGGAGAATTTTACAGAGCGGG TACAGTGACGGAGGAGCCTTCCGGAGTGTGAAGAACCTTTCCCCCAAAGA TGTGTG	

FIG. 2. Exon–intron structure. Exon and proximal intron sequences for the ubiquitously expressed exons within the Bin1 gene are shown. The figure is read from left to right with complete exon sequences shown on the *left* and introns following on the *right*. Register is 50 nt per line. Sizes of intron gaps are approximate based on estimations from restriction mapping.

gene is similar in both structure and organization to the human gene (Wechsler-Reya *et al.,* 1997b), including conservation of a large intron 1 (>20 kb) and a region of ~35 kb that includes the remaining exons. Exons were numbered by reference to the human gene. The mouse *Bin1* gene is ≥55 kb in length, which is similar to the ≥54-kb size of the human gene (Wechsler-Reya *et al.,* 1997b). A full-length mouse Bin1 message, which has been described [SH3P9; (Sparks *et al.,* 1996)], encodes the ubiquitously expressed Bin1 splice form Bin1-10 that includes exons 1–9, 11, and 13–16 (Wechsler-Reya *et al.,* 1997b,1998). The mouse and human coding sequences are ~89% identical at the nucleotide level (mouse nt 41–1461) and ~95% identical at the amino acid level (comparing the ubiquitously expressed Bin1-10 splice isoforms). Proximal intron sequences for each exon were generally highly conserved as well. The BAR (Bin1/Amphiphysin/Rvs167-related) region (Sakamuro *et al.,* 1996) encoded by exons 1–8 includes sequences that are conserved in amphiphysin and conserved in organization to the human Bin1 gene. Exons 9–11 encode the unique region of Bin1 that is not conserved in amphiphysin or RVS167. Exon 9 encodes the unique-1 (U1) region that includes sequences crucial for Bin1 to suppress malignant cell transformation by adenovirus E1A or mutant p53 (Elliott *et al.,* submitted for publication). Exon 10 encodes the unique-3 region (U3) of Bin1 which is alternately spliced following differentiation of C2C12 mouse myoblasts *in vitro* (Wechsler-Reya *et al.,* 1998). U3 was initially thought to encode a nuclear localization signal (Sakamuro *et al.,* 1996) but later investigations argued against this likelihood (Wechsler-Reya *et al.,* 1998). Exon 11 encodes the proline-rich unique-2 (U2) region, which can serve as a pseudosubstrate for the Bin1 SH3 domain (D. Sakamuro, unpublished observations). Murine exons corresponding to brain-specific exons 12A–12D in the human gene (Wechsler-Reya *et al.,* 1997b) were not analyzed in this study. Exons 13 and 14 encode sequences homologous to the Myc-binding domain in human Bin1 (Sakamuro *et al.,* 1996). Exon 13 is alternately spliced in an unregulated fashion in adult and embryonic mouse tissues (Wechsler-Reya *et al.,* 1998; Wechsler-Reya *et al.,* 1997b,).

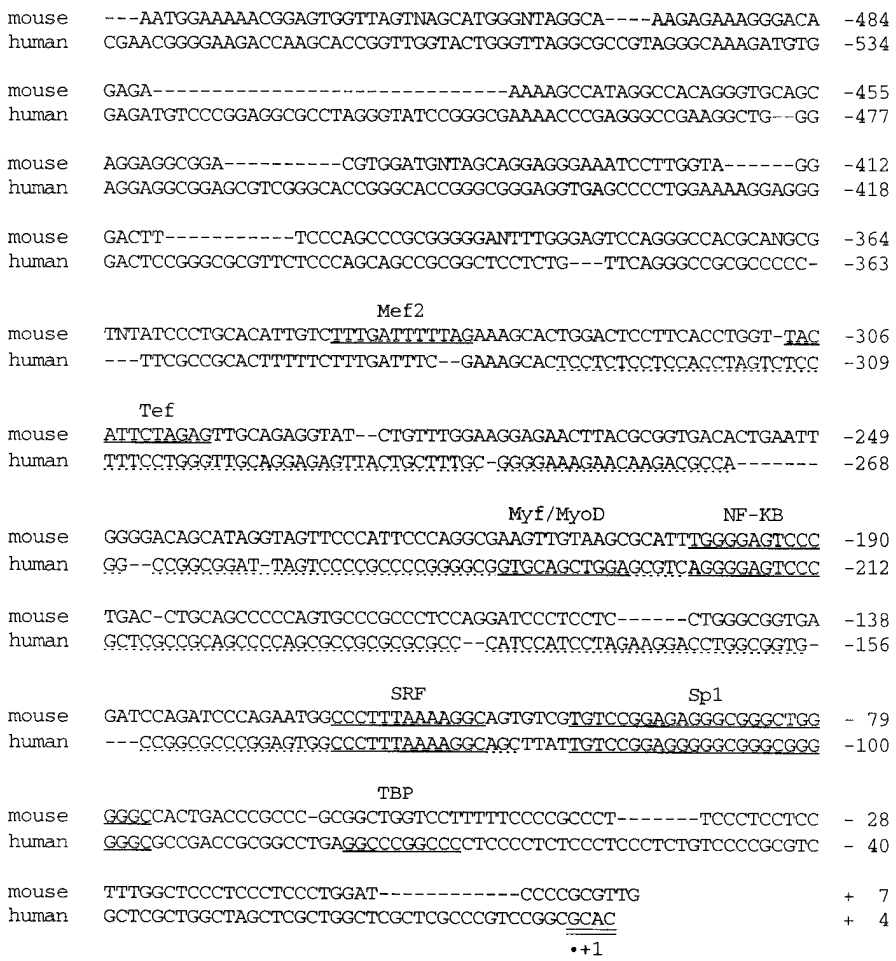

FIG. 3. Structure and conserved regulatory elements of the mouse Bin1 promoter. The DNA sequence of ~0.5 kb of the 5′ flanking region of the mouse gene is shown and aligned with the human promoter. Sequence alignment was performed using CLUSTAL W (1.7) except for the sequences immediately surrounding the human gene cap site (dot) and 5′ end of the mRNA (double underlining), which was aligned by visual inspection. Mouse sequences were numbered by comparison to the human promoter (Wechsler-Reya *et al.,* 1997b). A muscle regulatory module identified by a sequence analysis algorithm (Wasserman and Fickett, 1998) in the human promoter between −330 and −125 is indicated by dotted underlining. Consensus DNA binding sites for various transcription factors identified through this analysis or through visual inspection are noted by solid underlining. Mef2 and Tef consensus sites in the mouse were not conserved in the human promoter, whereas a strong Myf/MyoD consensus site in the human was not conserved in the mouse. A site analogous to the TBP binding site noted in the human promoter (Wechsler-Reya *et al.,* 1997b) was also not detected in the mouse sequence.

Exons 15 and 16 encode the Src homology 3 domain of Bin1, a feature that is shared with amphiphysin and RVS167 (Sakamuro *et al.,* 1996) and that is necessary for interaction with c-Abl and dynamin (Kadlec and Pendergast, 1997; Owen *et al.,* 1998).

Conserved Features of the Bin1 Promoter Suggest Roles in Myogenesis and Apoptosis

The DNA sequence of the 5′ flanking region upstream of exon 1 was determined, and this region was analyzed and compared with the human promoter (see Fig. 3). There was significant conservation between the mouse and the human 5′ flanking regions within 400 bp of exon 1, consistent with the identification of the mouse *Bin1* promoter. Bin1 is expressed robustly in skeletal muscle from adult mouse and human (Sakamuro *et al.,* 1996), so the 5′ flanking regions of each gene were analyzed using algorithms that identify transcription factor consensus binding sites and muscle regulatory modules (Wasserman and Fickett, 1998). A muscle regulatory module was identified in the human promoter between −330 and −125 (Fig. 3, dotted underline) but an analogous module was not detected in the mouse promoter. This absence suggested that there may be some difference in how the human and mouse genes are regulated during differentiation. Consistent with the latter possibility, the mouse region also lacked a CpG island that is present in the human promoter (Wechsler-Reya *et al.,* 1997b). The muscle regulatory sites identified in the human module included myogenin/myoD, Sp1, and serum response factor (SRF). The role of the myoD family b/HLH transcription factors in directing muscle differentiation is well known. SRF was initially identified as

an activator of the c-*fos* promoter but was later found to be crucial for regulating skeletal muscle-specific and smooth muscle-specific gene expression (Duprey and Lesens, 1994). In the 5′ flanking region of the mouse gene, the Sp1 and SRF sites were conserved but not the myogenin/myoD site, pointing to another difference with the human promoter. In place of the myogenin/myoD site were weak sites for Mef2 and Tef. Mef2 is a MADS-box transcription factor that is required for muscle development (Olson *et al.,* 1995). Since *Bin1* must be upregulated for differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), the Mef2 site may be relevant to this event because it has also been shown to be required for C2C12 differentiation (Ornatsky *et al.,* 1997). The Mef site may also be relevant to the high-level expression of *Bin1* in adult brain (Butler *et al.,* 1997; Sakamuro *et al.,* 1996; Wechsler-Reya *et al.,* 1997b) and in PC12 cells (R. Wechsler-Reya and G.C.P., unpublished results) since Mef2 isoforms are strongly expressed in neuronal cells (Lyons *et al.,* 1995). The Tef site may be relevant to housekeeping as well as muscle-specific regulation of Bin1. Tef was originally identified as an SV40 enhancer-binding factor but was subsequently discovered to be an important factor in cardiac muscle-specific gene regulation (Farrance *et al.,* 1992). Sp1 and Tef sites are proximal in the SV40 promoter so interactions between these factors may be involved in the basal but ubiquitous *Bin1* expression seen in tissues outside of muscle and brain (Wechsler-Reya *et al.,* 1997b). Taken together, the data suggested that SRF and Sp1 directed muscle-specific expression of *Bin1* with additional contributions from myogenin/myoD in human and from Tef and Mef2 in mouse.

One notable feature of the *Bin1* promoter identified in this study was the presence of an evolutionarily conserved strong consensus binding site for NF-κB. This finding was interesting because of evidence that Bin1 has a positive role in c-Myc-mediated apoptosis and that Bin1 can drive apoptosis of tumor cells that contain deregulated c-Myc (D. Sakamuro, K. Elliott, K. Ge, J. Duhadaway, D. Ewert, and G.C.P., manuscripts in preparation). NF-κB has important roles in oncogene-mediated cell transformation (Mayo *et al.,* 1997; Reuther *et al.,* 1998) and apoptosis (Baichwal and Baeuerle 1997). The likelihood that the NF-κB site in the Bin1 promoter is functional was supported by the observations that (1) Bin1 message levels were increased by TNF-α, which stimulates NF-κB activity, and that (2) a Bin1 promoter-reporter gene could be activated several-fold by TNF-α or RelA/p50 and c-Rel/p50 in transient cotransfection assays (data not shown). In future work, it will be important to determine whether Bin1 mediates certain NF-κB responses in apoptosis, for example, those activated by tumor necrosis factor, and whether the Myc-Bin1 system may modulate the ability of NF-κB to control apoptosis.

Bin1 Is Upregulated during Development by E10.5 in Myotomes

The inability to identify a muscle regulatory module in the mouse *Bin1* promoter prompted us to investigate the expression of *Bin1* during muscle development. Myogenesis initiates during development in somites, segmented paraxial mesoderm that is arrayed along the dorsal axis alongside the developing central nervous system. The dorsal part of the somite includes the myotome, which is the progenitor of skeletal muscle. Early stages of myogenesis are apparent in E10.5 myotomes because myf5 and myoD have been switched on and elongation of cells destined to become muscle can be seen. We performed an immunohistochemical analysis of E10.5 embryos with a Bin1 monoclonal antibody (Wechsler-Reya *et al.,* 1997a) to determine whether *Bin1* was switched on at this stage. In sagittal sections, strong staining was detected in elongated cells present in a dorsally located segmented pattern consistent with somites (see Figs. 4A and B). The cytoplasmic staining pattern was consistent with that seen following *in vitro* differentiation of C2C12 myoblasts, when Bin1 is exported from the nucleus to the cytosol (Wechsler-Reya *et al.,* 1998). In transverse sections, staining was confined to a medial part of a dorsolateral segment of the myotome (see Fig. 4C, arrowhead, and Fig. 4D). Staining was specific in the somite region inosfar as there was no significant staining of the adjacent sclerotomes, which are the progenitors of skeletal bone. The data indicated that *Bin1* was activated during myogenesis even though its promoter lacked a consensus DNA binding site for the important myoD family of muscle determination factors (Molkentin and Olson, 1996). Mef2 is expressed before E10.5 in the myotome (Edmondson *et al.,* 1994) so this factor may be responsible for activating the *Bin1* gene at this time. We concluded that despite the absence of a myoD family consensus binding site in its promoter, *Bin1* was activated at an early stage of myogenesis.

Bin1 Functions at an Early Time during Myoblast Differentation

In previous work, we showed that Bin1 is induced and has a necessary role during differentiation of C2C12 myoblasts (Wechsler-Reya *et al.,* 1998), a model for muscle differentiation *in vitro.* The immunochemical results above validated the induction of Bin1 seen in C2C12 and raised the question of when Bin1 acts during myoblast differentation. We have shown that C2C12 cells expressing antisense Bin1 do not exit the cell cycle and differentiate following serum deprival (Wechsler-Reya *et al.,* 1998). An important early event in C2C12 differentiation that leads to cell cycle inhibition is induction of the cell cycle kinase inhibitor $p21^{WAF1}$ (Walsh and Perlman, 1997). Therefore, we examined the regulation of $p21^{WAF1}$ or myosin, a marker for biochemical differentiation, in antisense or control C2C12 cell lines generated previously.

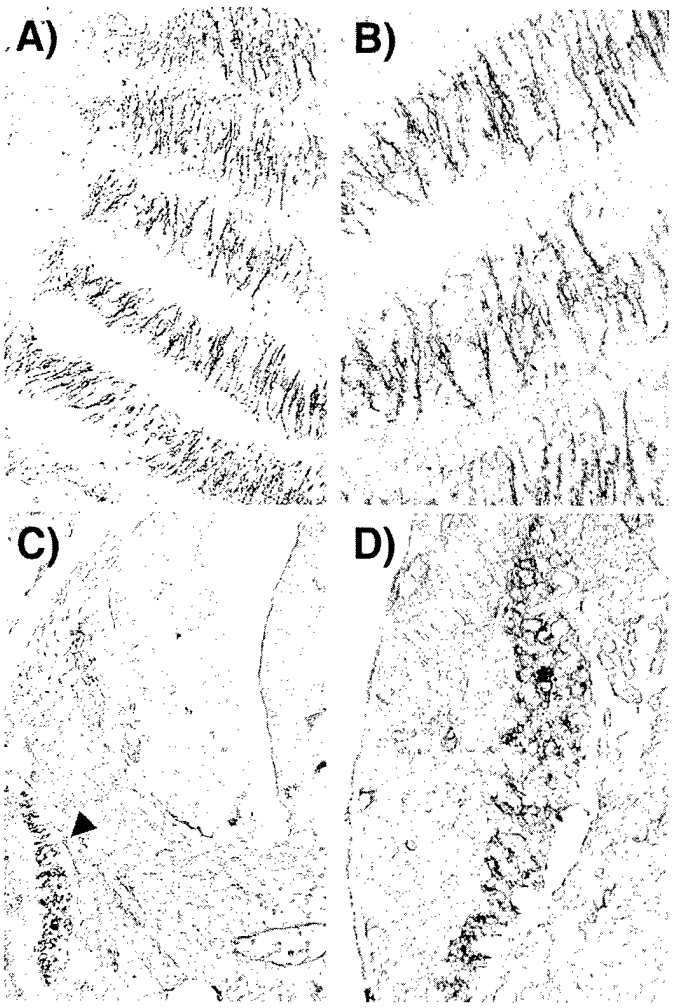

FIG. 4. *Bin1* is specifically upregulated in myotomes by E10.5 during murine development. E10.5 embryos were processed for Bin1 immunohistochemistry as described under Materials and Methods. (**A**) Sagittal section illustrating expression of *Bin1* in elongating myoblasts in somites, 80×. (**B**) Same section as above, 400×. (**C**) Transverse section illustrating expression of *Bin1* in the myotome but not the sclerotome of a somite (arrowhead), 80×. Orientation is provided by the neural tube seen in the upper right corner of the figure. Expression of *Bin1* in endothelial cells (bottom right side of the figure) and in certain neurons in the neural tube, a phenomenon also noted in the brain (data not shown), is also illustrated in this figure. (**D**) Same section as above, 400×.

Western analysis of extracts isolated at various times after induction of differentiation by serum deprival showed that $p21^{WAF1}$ was not appropriately upregulated in antisense cells (see Fig. 5). In control cells, $p21^{WAF1}$ levels steadily increased from a basal level of expression starting at day 1 after serum deprival. Myosin expression indicative of complete biochemical differentiation was first detected at day 3 in these cells. In contrast, in cells expressing antisense Bin1, $p21^{WAF1}$ was undetectable in undifferentated cells (day 0) and was only transiently induced on day 1 after differentiation was induced. As seen before, these cells did not proceed to express myosin and biochemically differentiate (Wechsler-Reya *et al.*, 1998), presumably because $p21^{WAF1}$ was not appropriately upregulated such that cells could exit the cell cycle. We concluded that Bin1 functioned at an early stage of myoblast differentiation, at a point required to sustain activation of $p21^{WAF1}$ and subsequent cell cycle exit.

Bin1 Is Located within the Proximal Region of Mouse Chromosome 18

The chromosomal location of *Bin1* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J × *M. spretus*)F_1 × C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 2500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). To identify informative RFLPs for gene mapping, the mouse *Bin1* cDNA was used as a probe in Southern blot analysis of C57BL/6J and *M. spretus* genomic DNAs digested with several restriction enzymes (see Materials and Methods). The inheritance of the *M. spretus*-specific alleles was followed in backcross mice, and the strain distribution pattern of the RFLP was determined to position the locus on the interspecific backcross map. The mapping results indicated that the *Bin1* locus is located in the proximal region of chromosome 18 (see Fig. 6), 0.5 cM proximal to *Apc*. The same results were obtained by using *Hin*cII polymorphisms and by using the human *BIN1* cDNA as a probe to follow *Taq* I polymorphisms (data not shown). We compared our interspecific map of chromosome 18 with composite mouse linkage maps that report the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME).

In humans, *BIN1* has been mapped to chromosome 2q14 by fluorescence *in situ* hybridization and by PCR analysis of somatic cell hybrids (Negorev *et al.*, 1996), but synteny has not been reported previously between human chromosome 2q14 and mouse chromosome 18. However, in the mouse, synteny has not been deter-

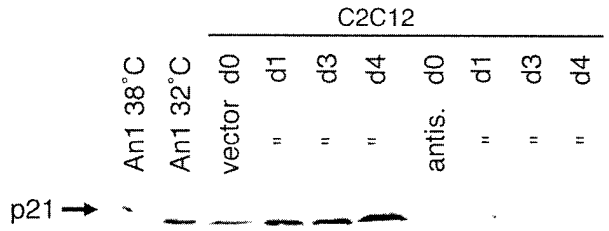

FIG. 5. Impaired activation of $p21^{WAF1}$ in myoblasts whose differentiation is blocked by antisense Bin1. Cell extracts were prepared from control or antisense Bin1-expressing C2C12 murine myoblasts (Wechsler-Reya *et al.*, 1998). Cells were cultured in growth medium (day 0, d0) or in differentiation medium for various times (d1,d3, and d4) before extract preparation. Western blotting was performed using anti-p21 and anti-myosin antibodies. A control for p21 induction was provided by the rat cell line BRK/An1, which harbors a temperature-sensitive p53 mutant; in this cell lines p21 is induced by activation of wildtype p53 at 32°C but not mutant at 38°C.

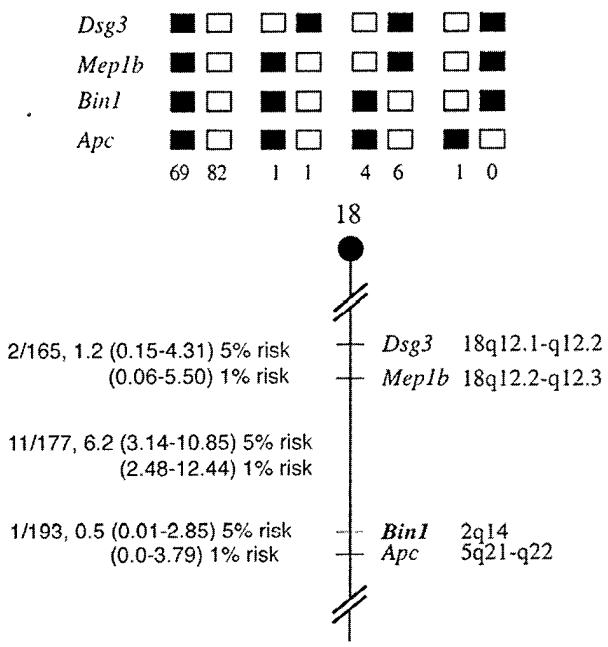

FIG. 6. Partial chromosome linkage map showing the mouse chromosomal location of *Bin1* as determined by interspecific backcross analysis. (**Top**) Segregation patterns of *Bin1* and flanking genes. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*)F_1 parent. Black boxes indicate the presence of a C57BL/6J allele, and white boxes indicate the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column, for a total of 164 mice analyzed for the segregation analysis of *Bin1*. (**Bottom**) Gene order analysis. Data from up to 193 mice were used to generate the partial chromosome linkage map of chromosome 18, which indicated the location of *Bin1* in relation to linked genes. To the left of the chromosome map is shown the number of recombinant N_2 animals over the total number of animals typed, with the recombination frequencies for each pair expressed as genetic distances in centimorgans (with confidence intervals at the 5 or 1% risk level). The positions of loci in human chromosomes are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

mined in the 6.7-cM region between loci that flank *Bin1* and have been mapped in both species (*Mep1b* and *Apc*). As discussed above, the human and mouse genes share significant similarity, and all the *Bin1* polymorphisms that we followed in backcross mice fell into the same region on mouse 18. Therefore, we conclude that the *Bin1* locus truly defines a new region of synteny.

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Bin1 functionally interacts with Myc and inhibits cell proliferation via multiple mechanisms

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The tumor suppressor Bin1 was identified through its interaction with the N-terminal region of Myc which harbors its transcriptional activation domain. Here we show that Bin1 and Myc physically and functionally associate in cells and that Bin1 inhibits cell proliferation through both Myc-dependent and Myc-independent mechanisms. Bin1 specifically inhibited transactivation by Myc as assayed from artificial promoters or from the Myc target genes ornithine decarboxylase (ODC) and α prothymosin (pT). Inhibition of ODC but not pT required the presence of the Myc binding domain (MBD) of Bin1 suggesting two mechanisms of action. Consistent with this possibility, a non-MBD region of Bin1 was sufficient to recruit a repression function to DNA that was unrelated to histone deacetylase. Regions outside the MBD required for growth inhibition were mapped in Ras cotransformation or HepG2 hepatoma cell growth assays. Bin1 required the N-terminal BAR domain to suppress focus formation by Myc whereas the C-terminal U1 and SH3 domains were required to inhibit adenovirus E1A or mutant p53, respectively. All three domains contributed to Bin1 suppression of tumor cell growth but BAR-C was most crucial. These findings supported functional interaction between Myc and Bin1 in cells and indicated that Bin1 could inhibit malignant cell growth through multiple mechanisms.

Keywords: c-Myc; transformation; tumor supressor; transcription

Introduction

Myc is a central regulator of cell proliferation and apoptosis that is frequently activated in human malignancy (reviewed in Henriksson and Lüscher, 1996; Prendergast, 1997; Facchini and Penn, 1998). In normal cells induced to divide, the levels of Myc increase and remain elevated, indicating it is required throughout the cell cycle for proliferation. Deregulated Myc expression is sufficient to drive quiescent cells into S phase to prevent cell cycle exit. Conversely, suppression of Myc blocks mitogenic signals and facilitates terminal differentiation. Myc can also induce apoptosis, a feature manifested in normal cells when its expression is enforced and uncoupled from the orchestration of other cell cycle regulatory events. Myc is thought to act in the guise of a transcription factor, but the exact mechanisms underlying its oncogenic and apoptotic properties remain obscure.

We previously identified a cellular polypeptide, Bin1, which interacts with the putative transcriptional activation domain of Myc (Sakamuro *et al.*, 1996). The interaction depends upon the integrity of the so-called Myc boxes, two evolutionarily conserved segments which are necessary for both cell transformation and apoptosis. Although its adaptor functions appear to be complex, several observations support the hypothesis that Bin1 is a tumor suppressor that controls cell cycle transit and proliferation. First, Bin1 inhibits cell transformation by Myc or adenovirus E1A (Sakamuro *et al.*, 1996). Second, Bin1 is related to RVS167, a negative regulator of the cell cycle in yeast (Bauer *et al.*, 1993). Third, although widely expressed in normal cells, Bin1 is poorly expressed or undetectable in ~50% of carcinoma cell lines and primary breast carcinomas examined (Sakamuro *et al.*, 1996). Fourth, deficits in expression are functionally significant, because Bin1 can inhibit the growth of tumor cells which lack endogenous expression (Sakamuro *et al.*, 1996). Fifth, similar to several other important tumor suppressors, Bin1 promotes differentiation in the myogenic pathway and its inhibition suppresses differentiation (Wechsler-Reya *et al.*, 1998). Finally, the human Bin1 gene has been mapped to chromosome 2q14 (Negorev *et al.*, 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher *et al.*, 1996), and recent investigations suggest that loss of Bin1 function may contribute to prostate tumor progression (unpublished observations). Evidence from genetic, *in vitro* biochemical association, and co-localization experiments supports interaction between Bin1 and Myc (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a) but *in vivo* physical association and functional interaction had not been documented. In addition, Bin1 was shown to inhibit growth by adenovirus E1A as well as Myc, but whether this reflected similar or different functions was undetermined. In this study, we show that Bin1 physically associates with Myc in cells and inhibits its transcriptional properties and that Bin1 can inhibit malignant cell growth through Myc-independent as well as Myc-dependent mechanisms.

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These findings support a role for Bin1 in governing the oncogenic properties of Myc but indicate that Bin1 also has additional roles in cell growth regulation.

Results

Physical and functional association of Myc and Bin1 in cells

Coimmunoprecipitation and transcription activation experiments were performed to examine the ability of Myc and Bin1 to functionally associate in cells. Association of Myc and Bin1 was observed to coimmunoprecipitation from baculovirus-infected Sf9 cells and untransfected C2C12 myoblasts, where Bin1 function has been examined (Wechsler-Reya *et al.*, 1998), using NP40 buffer conditions previously shown to support interaction of Myc and Bin1 *in vitro* (150 mM NaCl and 0.1% NP40). Bin1 was extracted more readily than Myc by NP40 lysis buffer from Sf9 cells infected with recombinant c-Myc and Bin1 baculoviruses, consistent with the fact that efficient extraction of Myc requires harsher conditions (RIPA buffer and sonication (Hann *et al.*, 1983). However, the Myc complexes extracted under these conditions contained Bin1 as indicated by coimmunoprecipitation with Myc antibody (Figure 1a). Association was specific because co-expression of the negative control proteins RhoB or yeast ADA3 with Bin1 did not result in Bin1 precipitation (data not shown). Bin1 antibodies capable to recognizing native Bin1 protein bind to epitopes in the Myc binding domain (MBD) (Wechsler-Reya *et al.*, 1997a) so the reverse immunoprecipitation experiment was intractable. Experiments using epitope-tagged Bin1 species were inconclusive, because tags at either the C- or N-terminus of Bin1 were not recognized unless denaturing conditions were used (i.e. RIPA buffer) that did not preserve Myc interaction *in vitro* (Sakamuro *et al.*, 1996; data not shown). However, Myc-Bin1 association was similarly observed in C2C12 cells. Myc and Bin1 are each expressed in proliferating C2C12 cells with Bin1 in stochiometric excess (Wechsler-Reya *et al.*, 1998). When C2C12 is induced to differentiate (Blau *et al.*, 1985), Bin1 is upregulated while Myc is downregulated to undetectable levels (Wechsler-Reya *et al.*, 1998), providing a useful negative control for association. As before, Myc was extracted inefficiently by NP40 buffer but Bin1 was detected in Myc complexes that were immunoprecipitated by Myc antibody (Figure 1b). The presence of Bin1 in these complexes was not due to antibody artifact or another nonspecific cause, because Bin1 was not detected in similar immunoprecipitates prepared from differentiated cell extracts.

To determine whether Bin1 association affected the transcriptional properties of Myc, transient activation assays were performed using a variety of promoters documented to be physiologically activated by c-Myc. The experiments employed luciferase (luc) reporter genes driven by artificial Myc-responsive promoters containing either multimerized DNA binding sites upstream of a minimal viral promoter or by the 5′ regions of the Myc target genes ornithine decarboxylase (ODC) and α-prothymosin (pT) (Bello-Fernandez *et al.*, 1993; Eilers *et al.*, 1991). The two artificial reporter genes were p3XMycE1b-luc (Gupta *et al.*, 1993) and Gal_5-E1b-luc, which included either three Myc-binding sites or five yeast GAL4 binding sites upstream of the adenovirus E1b minimal promoter. The latter reporter was used where activation was driven by chimeric molecules containing Bin1 or the Myc N-terminal transactivation domain (aa 1–262) fused to the DNA binding domain of the yeast transcription factor GAL4 (Kato *et al.*, 1990). The ODC and target gene reporters were ODCΔluc and PrT-luc (Bello-Fernandez *et al.*, 1993; Desbarats *et al.*, 1996; Packham and Cleveland, 1997). Cells were transfected with reporter plasmids and vectors for c-Myc or GAL4-Myc, Bin1, or the MBD deletion mutant Bin1ΔMBD (Sakamuro *et al.*, 1996). Max was included in pT experiments for optimal activation of this gene as documented (Desbarats *et al.*, 1996). Western or Northern analyses confirmed exogenous gene expression in transiently transfected cells (data not shown). ODC activation experiments included as a positive control for N-terminal interaction and inhibition of Myc activation the retinoblastoma (Rb)-

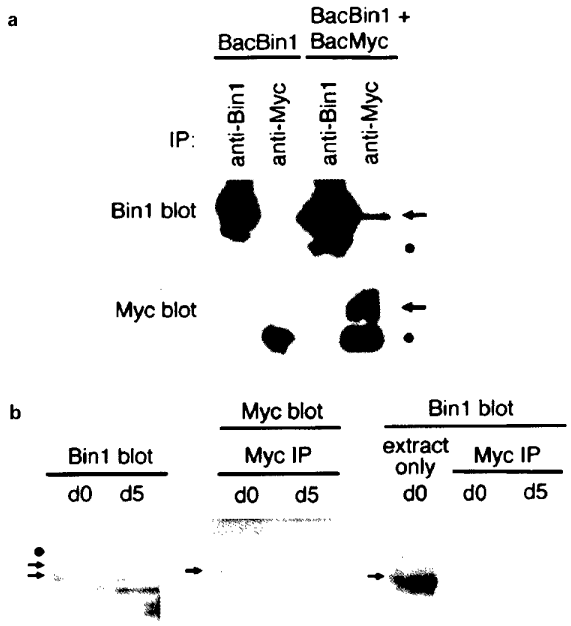

Figure 1 Biochemical association of Bin1 and Myc in cells. (**a**) Association in Sf9 cells. Extracts from 2×10^6 cells infected with the recombinant baculoviruses indicated were prepared and subjected to IP/Western analysis as described in the text and the Materials and methods. Dots indicate the position of coprecipitating antibodies recognized by anti-mouse or anti-rabbit secondary antibodies used to develop the blots, by a chemiluminescence technique. (**b**) Association in naive C2C12 myoblasts. Extracts from growing (d0) or differentiated (d5) C2C12 cells were prepared and subjected to Western or IP/Western analyses as described in the text and the Materials and methods. The left panel is a Western blot of an SDS gel loaded with 50 μg extract from d0 or d5 cells, demonstrating constitutive Bin1 expression and the appearance of a larger alternately spliced species in differentiated cells (Wechsler-Reya *et al.*, 1998).The dot indicates a nonspecific band. The middle panel is a Western blot of nonreducing SDS gel loaded with a Myc immunoprecipitate (sc-42) generated from 1.5 mg of d0 or d5 extracts and probed with a second anti-Myc antibody (9E10). The right panel is a Western blot of a nonreducing SDS gel loaded with 50 μg of d0 extract alone or a Myc immunoprecipitate (sc-42) from 1.5 mg d0 or d5 extracts and probed with anti-Bin1 99D

related protein p107 (Beijersbergen *et al*., 1994; Gu *et al*., 1994).

Bin1 selectively inhibited Myc activation on all the Myc reporter promoters tested (Figure 2). In NIH3T3 cells, Myc activated p3XMycE1b-luc ~2.5-fold, similar to the level observed by others (Kretzner *et al*., 1992), and titration of Bin1 into the assay reversed the effect of Myc (Figure 2a). Similarly, Myc activated the ODC promoter ~2.3-fold, also as documented previously (Packham and Cleveland, 1997), and Bin1 reversed this effect as potently as p107 (Figure 2b). Deletion of the Myc-binding domain (MBD) from Bin1 relieved its ability to inhibit ODC in both HeLa and NIH3T3 cells (Figure 2c). The inability of Bin1ΔMBD to suppress Myc was not due to polypeptide instability nor to general loss of function, because Bin1ΔMBD accumulated similarly to wild-type Bin1 in transfected COS cells and because Bin1ΔMBD could inhibit E1A transformation (see below). A more robust activation of pT by Myc-Max was also inhibited by Bin1 ~3-fold (Figure 2d). Bin1ΔMBD also inhibited Myc activation of pT indicating the effect on this gene was MBD-independent. However, inhibition was specific because Bin1 did not affect activation by VP16. Western analysis confirmed Myc and Max accumulation in transiently transfected cells, ruling out the trivial possibility that Bin1 acted by inhibiting the exogenous Myc or Max expression (data not shown). The specificity of the effect of Bin1 for the Myc N-terminus was investigated using GAL4-Myc or a second GAL4 chimera which included instead the activation domain from the nonspecific but broadly active herpes virus activator VP16 (GAL4-VP16). For these experiments, we examined activation of a pT reporter (GAL4mE-prT-luc) that was identical to the prT-luc reporter used above except that the two Myc

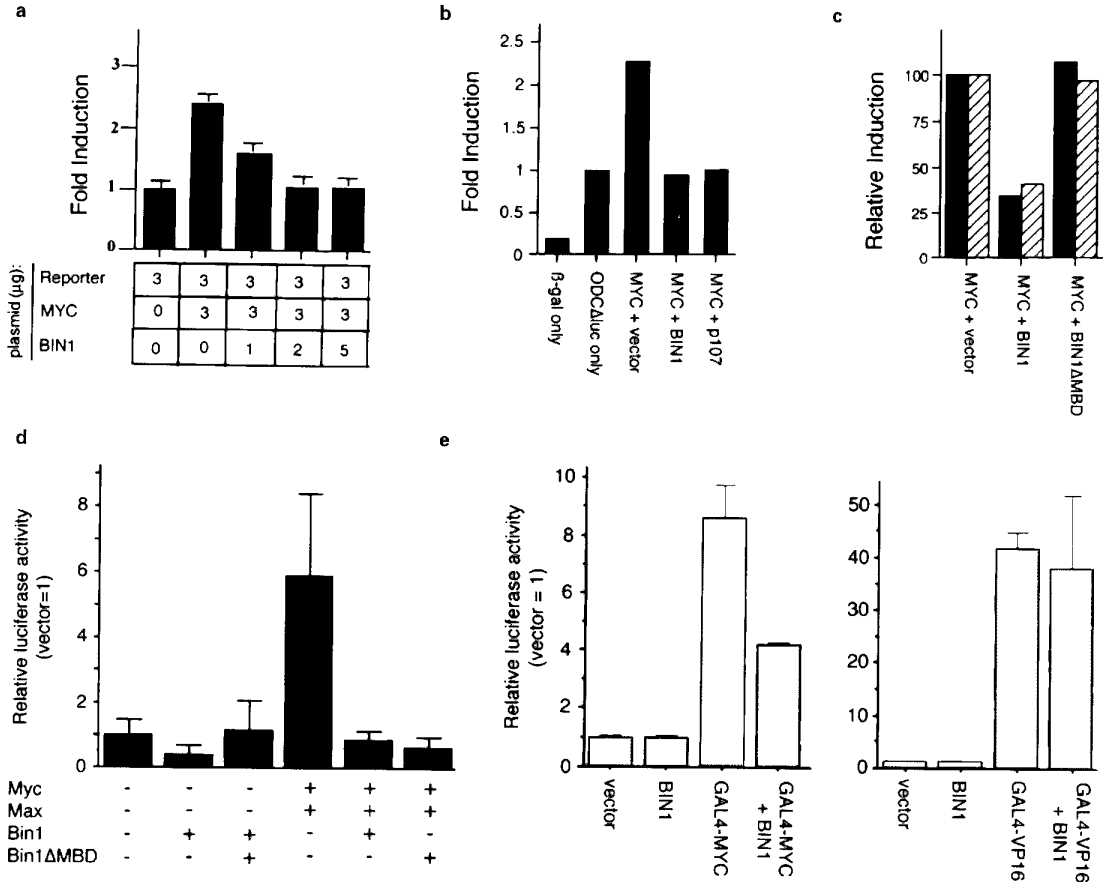

Figure 2 Bin1 specifically inhibits gene activation by Myc. (**a**) Inhibition of a Myc-responsive artificial promoter. NIH3T3 cells were transfected with the plasmids indicated by standard calcium phosphate method and processed for normalized luciferase activity as described (Zhang and Prochownik, 1997). The data represent the results of three trials each performed in duplicate. (**b**) Inhibition of ODC activation. NIH3T3 cells seeded into six well dishes were transfected with 1.5 μg of the ODC reporter ODCΔluc, 3 μg of the human c-Myc vector LTR Hm, 1.5 μg CMV Bin1 or CMV p107, and 0.5 μg CMV-βgal (to normalize for transfection efficiency). pcDNA3 was added to equalize the amount of plasmid in each transfection. Two days later cell extracts were prepared and processed for normalized reporter activity. The graph depicts relative luciferase activity based on reporter only (set at 100%); the absolute values ranged from 10^3-10^4 light units. The results represent the average of two trials each performed in duplicate. (**c**) MBD is required for ODC inhibition. NIH3T3 or HeLa cells were transfected with 0.5 μg ODCΔluc and 2 μg LTR-Hm plus 3.25 μg vector, CMV-Bin1, or CMV-Bin1ΔMBD. Cell extracts were prepared and processed as above. The results represent the average of two trials performed in duplicate. Relative luciferase activity is depicted as the proportion of reporter plus LTR Hm; the absolute values ranged from 10^3-10^4 light units. (**d**) Inhibition of pT activation. HeLa cells were transfected with PrT-luc, a β-galactosidase normalization plasmid, and the vectors indicated as described (Desbarats *et al*., 1996). Where indicated Bin1 or control plasmids were included in a 1:1 w/w ratio with Myc. Relative luciferase activity is depicted as above; the absolute values ranged from 10^4-10^6 light units. (**e**) Bin1 inhibits GAL4-Myc but not GAL-VP16. HeLa cells were transfected with GAL4mE-PrT-luc and the genes indicated as above and processed for relative luciferase activity

binding sites in the gene were replaced with GAL4 binding sites (Desbarats *et al.*, 1996). Bin1 inhibited activation of pT by GAL-Myc but not by GAL4-VP16 (Figure 2e). Similar results were obtained with GAL4-E1b-luc (data not shown). Taken together, the results of the immunoprecipitation and transcription experiments argued that Bin1 physically and functionally interacted with Myc in cells.

Bin1 can recruit a transcriptional repression function to DNA

Bin1 does not harbor motifs characteristic of transcription adaptor proteins, so one interpretation of the above results was that Bin1 acted via a passive mechanism, for example, by occluding contacts with as yet unidentified coactivators or with the TATA-binding protein (TBP), which has been reported to interact with Myc (Hateboer *et al.*, 1993). Alternately, Bin1 may act through an active repressive mechanism, perhaps by recruiting a corepressor to the promoter similar to the Mad-binding protein mSin3 (Ayer *et al.*, 1995; Schreiber-Agus *et al.*, 1995). To assess the latter hypothesis, we tested the effects of Bin1 on basal transcription of a promoter to which it was tethered in a Myc-independent manner. This was achieved by fusing Bin1 in frame to the DNA binding domain of GAL4 to generate GAL4-Bin1. A second GAL4 chimera that lacked the MBD was constructed (GAL4-Bin1ΔMBD) to eliminate MBD-dependent interactions with Myc, Myc-binding coactivators yet to be identified, or possibly TBP (Hateboer *et al.*, 1993), all of which might mask repressive effects or make their interpretation more difficult. HeLa cells were transfected with the artificial promoter-reporter gene GAL_5-E1b-luc and equivalent amounts of expression vectors for unfused GAL4 DNA binding domain (GAL0), GAL4-Bin1, or GAL4-Bin1ΔMBD and cell lysates were processed for luciferase activity as before. GAL4-Bin1 was only slightly inhibitory but GAL4-Bin1ΔMBD repressed basal transcription ~2.5-fold relative to unfused GAL0 (Figure 3a). GAL4-Bin1ΔMBD had little effect on the activity of luciferase reporters lacking GAL4 sites (data not shown), indicating that this effect was dependent on DNA binding. To determine if repression reflected recruitment of a Bin1-binding factor, we added vector, wild-type (untethered) Bin1, or Bin1ΔMBD to the cotransfection. If the activity was intrinsic, cotransfection of Bin1 would not affect repression, whereas if repression was due to recruitment of a *trans*-acting factor then untethered Bin1 would be predicted to titer the repressive effect. Consistent with the latter case, both Bin1 and Bin1ΔMBD relieved repression by GAL4-Bin1ΔMBD (Figure 3b). The greater relief provided in Bin1ΔBD suggested that a region outside of the MBD might recruit a repression function. Experiments in which trichostatin A was added did not relieve the repressive effect of GAL4-Bin1ΔMBD suggested that a region outside of the MBD might recruit a repression function. Experiments in which trichostatin A was added did not relieve the repressive effect of GAL4-Bin1ΔMBD (data not shown), suggesting that this function was not a histone deacetylase and that Bin1 acts differently than mSin3 (Facchini and Penn, 1998). Nevertheless, the results suggested that Bin1 may actively inhibit Myc activation by recruiting a repression function.

Expression and localization of Bin1 deletion mutants

To identify non-MBD regions that are important for Bin1 activity a set of deletion mutants was constructed

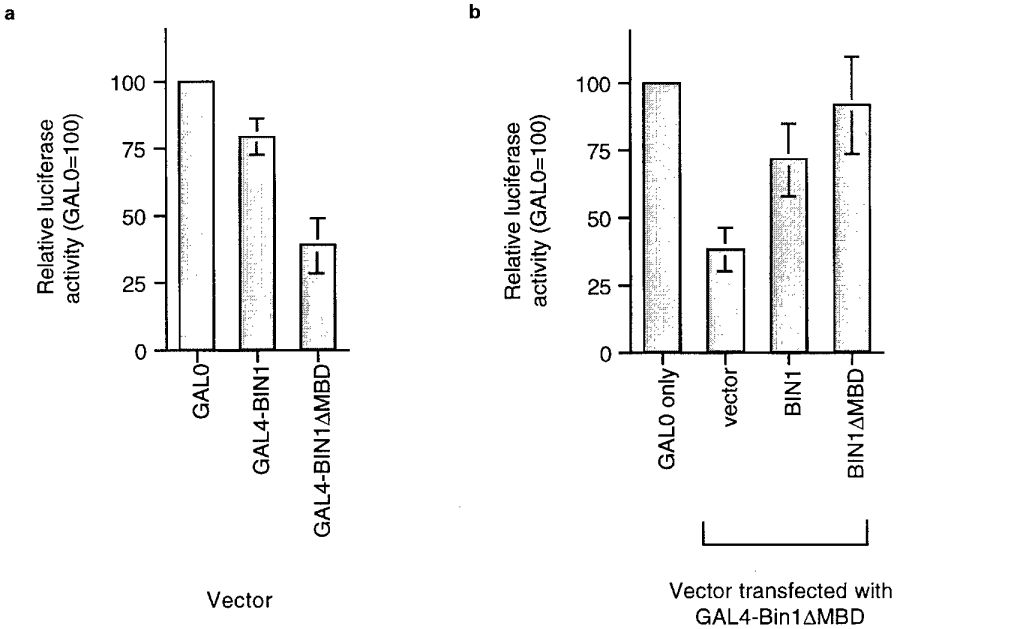

Figure 3 Bin1 recruits a repression function when tethered to a promoter. (**a**) Intrinsic repressive quality of Bin1. HeLa cells were transfected with 2 μg GAL4-SV40-luc reporter and 4 μg of the indicated GAL4 chimeric gene and normalized luciferase activity was determined 2 days later. The data represent the results of at least four trials each performed in duplicate. (**b**) The repressive activity of BIN can be titered. Cells were transfected with 2 μg GAL4-SV40-luc reporter, 4 μg of GAL4-Bin1ΔMBD, 4 μg CMV vector, Bin1, or Bin1ΔMBD plasmids and normalized luciferase activity was determined 2 days later. The data represent the results of four trials each performed in duplicate

(Figure 4). BAR-C and SH3 encompass regions of Bin1 that are related to the neuron-specific protein amphiphysin and to the yeast cell cycle regulator RVS167 (the BAR nomenclature reflects the Bin1/

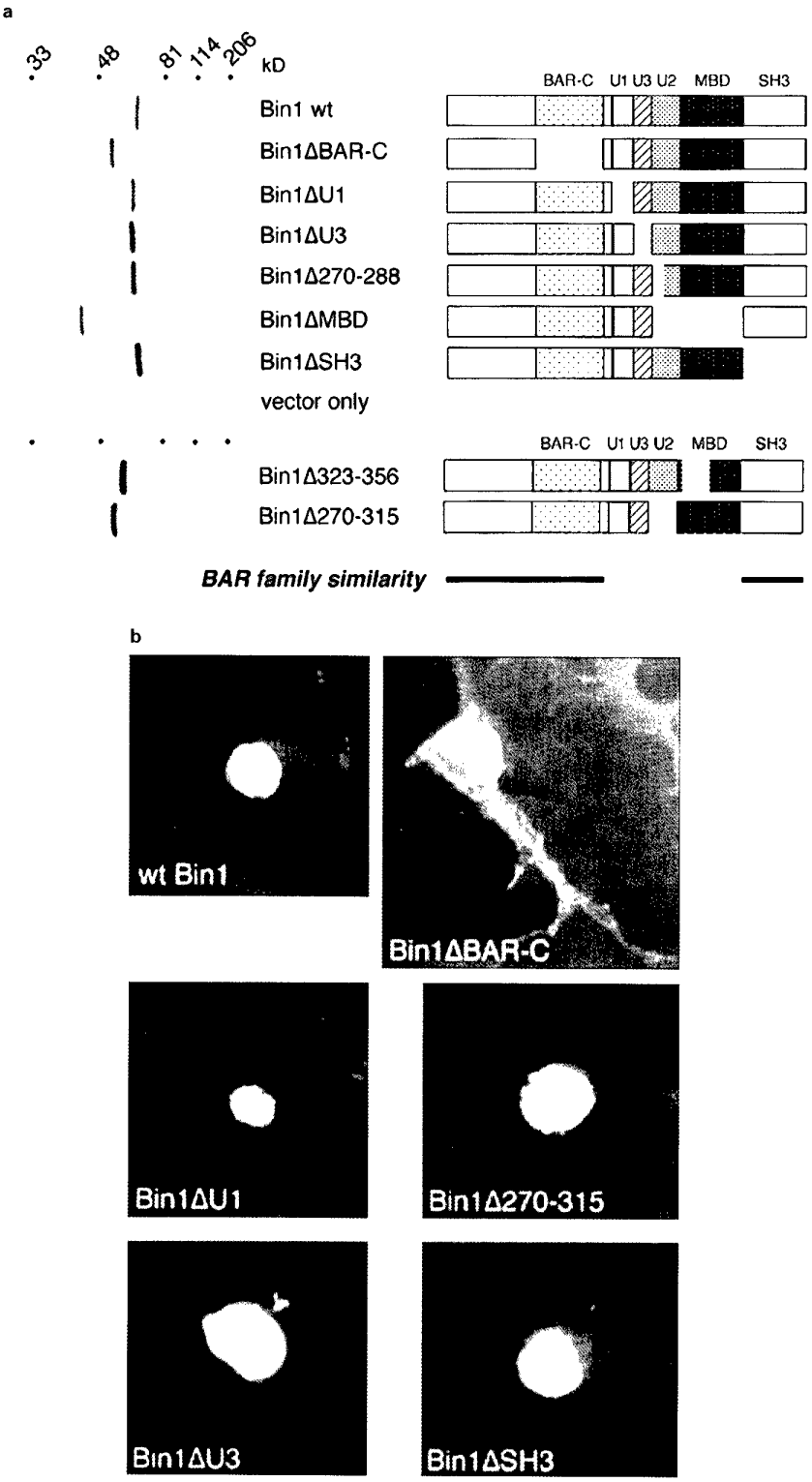

Figure 4 Structure, expression, and localization of Bin1 deletion mutants. (**a**) Expression of Bin1 mutants. COS cells were transfected with the expression vectors indicated, metabolically labeled with ^{35}S-methionine/cysteine, and cell extracts were prepared and subjected to immunoprecipitation with Bin1 monoclonal antibodies. Immunoprecipitates were examined by SDS–PAGE and fluorography. The bars at the bottom of the cartoon denote regions that are structurally related among proteins of the BAR family, which includes Bin1, amphiphysin, and RVS167 (Sakamuro *et al*., 1996). (**b**) Localization of Bin1 mutants. 293T cells seeded on glass cover slips were transiently transfected with the expression vectors and processed for indirect immunofluorescence with Bin1 monoclonal antibody 99D as described in the Materials and methods

amphiphysin/RVS167 homology in this region; BAR-C represents the C-terminal half of the BAR domain (Figure 4a). The SH3 domain located at the C-terminus is dispensable for interaction with Myc (Sakamuro *et al.*, 1996). The central region is not conserved in amphiphysin or RVS167 and is unique to Bin1. This region includes the so-called unique-1 (U1) region encoded in the human gene by exon 9; the alternately spliced and strongly positively charged unique-3 (U3) region encoded by exon 10; the unique-2 (U2) region encoded by exon 11 which harbors two copies of the SH3 binding motif PXXP; and the MBD (Wechsler-Reya *et al.*, 1997b). The MBD as initially defined encompassed aa 270–389. Deletions of three subsections of this segment were generated for this study, aa 270–288, aa 270–315 (comprising the newly defined U2 region) and aa 323–356 (N-terminal half of the MBD). Expression of the mutant polypeptides was confirmed by immunoprecipitation from COS cell extracts. Cells were transfected with vectors for each mutant, metabolically labeled with 35S-methionine, and extracts were prepared and processed for immunoprecipitation with a mixture of Bin1 monoclonal antibodies (Wechsler-Reya *et al.*, 1997a). The apparent and predicted MWs of the mutants did not coincide in each case because of the presence of a determinant for aberrant gel mobility that maps to the MBD region (Sakamuro *et al.*, 1996). Each mutant was observed to accumulate as efficiently as full-length Bin1 (Figure 4a). The cell localization of several mutants was examined by indirect immunofluorescence in transiently transfected 293T cells (Figure 4b). The presence of an SV40 replication origin on the expression vectors made it possible to distinguish cells expressing exogenous proteins by using a higher dilution of Bin1 monoclonal antibody than needed to detect endogenous expression (1 : 100 instead of 1 : 5 dilution). Consistent with previous results (Sakamuro *et al.*, 1996; Wechsler-Reya *et al.*, 1997a), wild-type Bin1 localized exclusively to the nucleus, as did Bin1 mutants lacking the U1, U2, U3 and SH3 regions (Bin1ΔU1 was also preferentially excluded from the nucleolus). U3 contains a nuclear localization motif but its dispensability for nuclear localization was consistent with recent findings in which alternate splicing of the exon encoding U3 after myoblast differentiation is correlated with the appearance of cytosolic Bin1 species (Wechsler-Reya *et al.*, 1998; Wechsler-Reya *et al.*, 1997b). Instead, BAR-C contained a critical nuclear localization signal, because both nuclear and cytosolic staining was detected in cells transfected with Bin1ΔBAR-C. We concluded that BAR-C sequences between aa 125–207 included signal(s) for nuclear localization and/or retention.

Bin1 inhibits malignant cell transformation by multiple mechanisms

Using the Ras cooperation assay performed in primary rat embryo fibroblasts (REFs) (Land *et al.*, 1983; Ruley, 1983), we previously showed that Bin1 inhibits malignant transformation by c-Myc in a MBD-dependent manner (Sakamuro *et al.*, 1996). To define other regions required, REFs were transfected with expression vectors for Myc, oncogenic Ras, and Bin1 or Bin1 deletion mutants, and transformed cell foci were scored 2 weeks later (Figure 5). Consistent with previous results (Sakamuro *et al.*, 1996), wild-type Bin1 suppressed focus formation by Myc ~sixfold relative to the empty vector control. Most deletion mutants inhibited focus formation as efficiently as wild-type Bin1, suggesting modularity in the structural organization of this polypeptide. Only BAR-C or the MBD segment aa 323–356 were required, identifying BAR-C determinants as crucial to inhibit Myc transformation along with the MBD. Since aa 270–315 (U2 region) was dispensable for inhibiting Myc transformation the critical part of the MBD therefore was confined to a 66 residue segment between aa 323–389. The inactivity of the MBD aa 323–356 or BAR-C deletion mutants was not due to protein instability, because each polypeptide accumulated similar to wt Bin1 in COS cells (Figure 4a), nor to misfolding, because each polypeptide efficiently suppressed transformation by E1A or mutant p53. Bin1ΔBAR-C localized to the nucleus and cytoplasm (Figure 4b) but its ability to suppress E1A and mutant p53, which act in the nucleus, also argued against mislocalization as the cause for loss of activity against Myc. We previously showed that Bin1 inhibited transformation by adenovirus E1A but not SV40 large T antigen (Sakamuro *et al.*, 1996), and in this study we show that Bin1 also inhibited transformation by dominant inhibitory mutant p53. Bin1 suppressed transformation by E1A or mutant p53 ~threefold (Figure 6); the inhibitory effects of each could be titered as was the case with Myc (Sakamuro *et al.*, 1996) by altering the ratio of Bin1 to E1A or mutant p53 in the assay (data not shown). U1 was crucial to inhibit E1A and U1 and SH3 were both crucial to inhibit mutant p53 (Figure 6). U3, BAR-C, and MBD were each dispensable to inhibit either oncoprotein. As before, neither protein instability nor misfolding was responsible for the loss of activity of either mutant since each accumulated in COS and each could suppress Myc transformation (Figures 4a and 5).

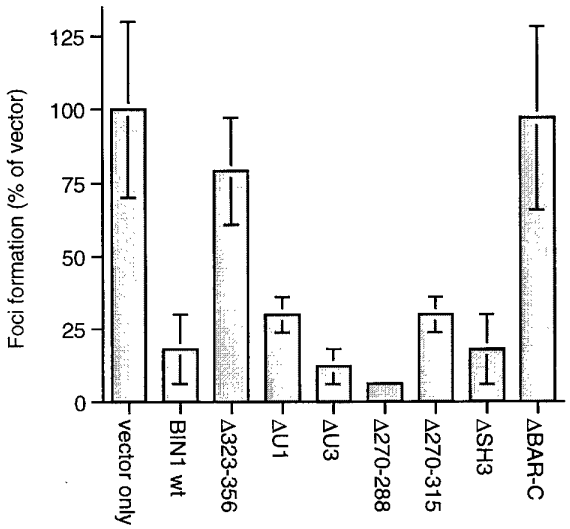

Figure 5 BAR-C is required to inhibit Myc transformation. REFs were transfected with 5 μg each oncogenic Ras and deregulated human c-Myc plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12–14 days later. The data depict the percentage of Myc + Ras foci formed in the presence of empty vector

Northern analyses of RNA isolated from pools of foci derived from Myc+Ras, E1A+Ras, or mutant p53+Ras transfections showed that, as predicted, mutant Bin1 messages accumulated in transformed cells if the mutant was biologically inactive. For example, Bin1ΔBAR-C message only accumulated in Myc+Ras foci whereas Bin1ΔU1 message only accumulated in E1A+Ras or mutant p53+Ras foci (data not shown). Thus, the domains required to inhibit E1A and mutant p53 were distinct from those required to block Myc. The importance of the BAR-C domain to the inhibitory activity of Bin1 was confirmed in HepG2 cells (Figure 7). Deletion of other domains only partly relieved HepG2 growth consistent with the likelihood that multiple growth mechanisms were deregulated in these tumor cells (data not shown). Notably, MBD deletion also only slightly relieved suppression, underscoring the importance of MBD-independent mechanisms for some types of growth inhibition by Bin1. Since neither E1A nor mutant p53 require endogenous Myc to transform cells, the differences in domain dependence argued that Bin1 could regulate malignant cell proliferation through Myc-independent as well as Myc-dependent mechanisms.

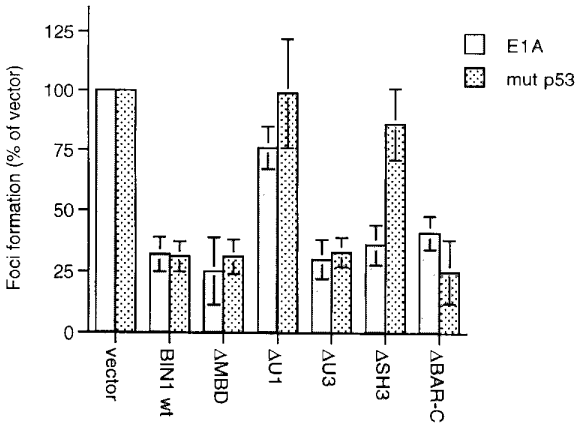

Figure 6 U1 is required to inhibit E1A transformation and U1 and SH3 are each required to suppress mutant p53 transformation. REFs were transfected with 5 μg each oncogenic Ras and adenovirus E1A or dominant inhibitory p53 mutant plasmids plus 10 μg each of the vectors indicated. Transformed cell foci were scored 12–16 days later. The data depict the percentage of E1A+Ras or mutant p53+Ras foci formed in the presence of empty vector

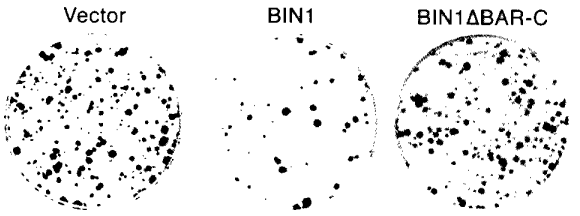

Figure 7 BAR-C is crucial to suppress tumor cell growth. HepG2 cells were transfected with 2 μg of neomycin (neo^r)-resistance gene marked vectors. G418-resistant cell colonies were scored ~3 weeks later by methanol fixation and cystal violet staining. A representative assay is shown from multiple experiments performed in triplicate

Discussion

This study supports the assertion that Myc and Bin1 physically and functionally associate in cells, and it showed that Bin1 can inhibit malignant cell proliferation by both Myc-dependent and Myc-independent mechanisms (Figure 8). Myc-Bin1 complexes were detected by coimmunoprecipitation from recombinant baculovirus-infected Sf9 cells or from naive C2C12 cells. The fact that Myc-Bin1 complexes could be identified in growing C2C12 cells suggested that association is not inhibitory *per se* but may be subjected to posttranslational regulation. This possibility would be consistent with demonstrations that Bin1 is phosphorylated and associated *in vivo* with other proteins in addition to Myc (Wechsler-Reya *et al*., 1997a). The ability of Bin1 to specifically inhibit Myc function as measured by activation of artificial and natural target genes supported *in vivo* association. Activation by Myc/Max or by GAL4-Myc chimeras containing the Myc transactivation domain, but not by GAL4-VP16, was susceptible to Bin1 inhibition. VP16 is a complex activator that can act through a variety of adaptors, so the fact that VP16 was not inhibited by Bin1 indicated that its activity was specific and not due to nonselective suppression of transcriptional activation. ODC and pT are two paradigm target genes for Myc and the ability of Bin1 to inhibit each supported the notion of functional interaction. Whether Bin1 has

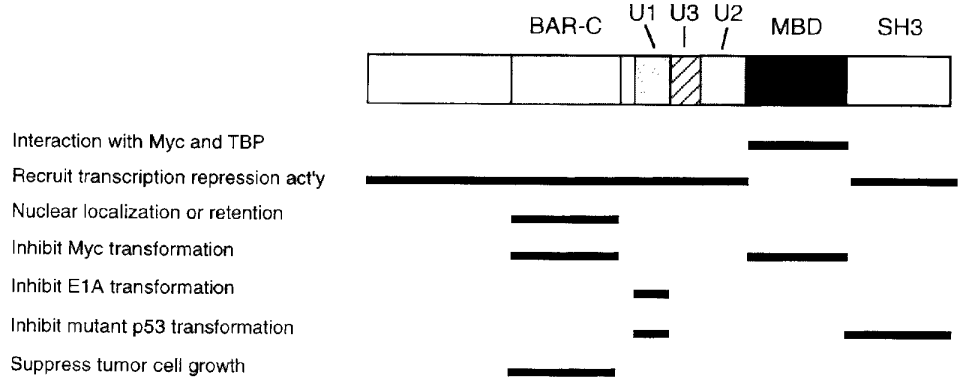

Figure 8 Summary of Bin1 functions. Myc interaction data is from Sakamuro *et al*. (1996)

a physiological role in transcription bears further analysis. However, in support of this possibility we showed that Bin1 could recruit a potential repression function to DNA via an MBD-independent interaction. In addition, Myc has been reported to interact *in vitro* with TATA-binding protein (Hateboer *et al.*, 1993) and we have observed that the Bin1 MBD can avidly bind TBP *in vitro* (D Sakamuro and GC Prendergast, unpublished observations). Although the consequences of Myc-TBP interaction have not been established *in vivo* the ability of Bin1 to bind TBP conceivably represents a second mechanism through which Bin1 could disrupt Myc activation. As considered above, it is possible that the inhibitory effects of Bin1 on Myc activation are passive and an epiphenomenon of steric occulusion of coactivators which are yet to be identified. Recent results indicated that Bin1 is necessary for Myc-mediated apoptosis (D Sakamuro, J Duhadaway and GC Prendergast, unpublished observations) would provide a biological foundation to assess the physiological significance of the putative transcriptional properties of Bin1 documented in this study.

The N-terminal BAR-C region of Bin1 was required to inhibit Myc transformation. BAR-C is a charged region of 84 aa predicted to be both α helical and involved in coiled-coil interactions (Lupas, 1996). A key functional role for this region is supported by the fact that it contains the most highly conserved sequences in Bin1 in evolution. Given the requirement for MBD and BAR-C to suppress Myc transformation one might have expected both regions to be important for the inhibitory effects of Bin1 in HepG2, which overexpresses Myc. However, if Myc-independent growth pathways deregulated in HepG2 are dominant or co-dominant with Myc-dependent pathways then this would not be expected to be the case. BAR-C included a signal(s) for nuclear localization or retention, while NLS-like sequences in U3 (Sakamuro *et al.*, 1996) have been shown here and elsewhere (Wechsler-Reya *et al.*, 1998) to be dispensable. The results of this study mapped the MBD within a 61 residue segment between aa 315–376 immediately upstream of the SH3 domain. Interestingly, this region of Bin1 is encoded by two exons and the more 5′ exon has been found to be alternately spliced in cells (Wechsler-Reya *et al.*, 1997b). The aa 323–356 deletion which relieved Myc suppression activity closely overlaps the sequences encoded by this exon. Thus, one alternately spliced Bin1 species in cells probably lacks Myc binding capacity and functions independently of Myc, a likelihood that is consistent with Myc-independent growth inhibitory properties of Bin1 identified in this study. Alternative splice forms of Bin1 that are neuron-specific, termed amphiphysin-like isoform or amphiphysin II, have been implicated in endocytosis (Wigge and McMahon, 1998). However, we do not believe endocytosis is relevant to the Myc-independent growth inhibition mechanisms identified here, because non-neuronal splice forms lack determinants required for interaction with endocytosis systems (Ramjaun and McPherson, 1998) and because the inclusion of neuron-specific exons in Bin1 eliminates its growth inhibitory activity (unpublished observations).

The C-terminal U1 and SH3 regions were required to inhibit transformation by E1A or p53 but not by Myc. U1 is contained on a single exon which encodes 28 aa (Wechsler-Reya *et al.*, 1997b). E1A transforms cells by displacing E2F from Rb (Dyson and Harlow, 1992) so U1 either impedes this process somehow or acts downstream to interfere with E2F effectors. Consistent with a link between U1 and the Rb/E2F system, U1 deletion also blocks transformation by the human papilloma virus E7 protein (data not shown), which acts similarly to E1A by interfering with Rb/E2F interaction (Phelps *et al.*, 1988). The requirement of U1 to inhibit mutant p53 is consistent with evidence that cell transformation by mutant p53 also depends on interference with Rb/E2F interactions (Hansen *et al.*, 1995). The SH3 domain of Bin1 was also necessary to inhibit transformation by mutant p53. To our knowledge Bin1 and Abl are the only two SH3-containing proteins localized to the nucleus, and recently Abl has been shown to interact with Bin1 in an SH3-dependent manner (Kadlec and Pendergast, 1997; D Sakamuro and GC Prendergast, unpublished observations). This may be of consequence since Abl and p53 have been reported to interact in cells (Yuan *et al.*, 1996), although the physiological significance of this interaction has not been established clearly. Direct interaction between the Bin1 SH3 and the PxxP motifs in the apoptosis effector domain of p53 (Sakamuro *et al.*, 1997) could be germane since PxxP motifs constitute SH3 ligands. Indeed, since this region also has been implicated in growth inhibition (Walker and Levine, 1996) and the transforming efficiency of mutant p53 rests upon more than simple inactivation of endogenous p53 (Dittmer *et al.*, 1993; Hulboy and Lozano, 1994), it is conceivable that mutant p53 may promote transformation in a PxxP-dependent manner by sequestering a nuclear SH3-containing growth suppressor such as Bin1.

Materials and methods

Plasmid constructions

The following plasmids have been described. CMV-Bin1 and CMV-Bin1ΔMBD encode full-length Bin1 or an MBD deletion mutant, respectively (Sakamuro *et al.*, 1996). LTR Hm contains a Moloney retroviral long terminal repeat-driven normal human c-*myc* gene (Kelekar and Cole, 1986); pSVLneo-C-myc is an SV40 early region-driven c-Myc vector used in Figure 2a that has been described (Zhang and Prochownik, 1997); p1A/neo contains the 5′ end of the adenovirus type 5 genome including the E1A region (Maruyama *et al.*, 1987); LTR p53ts encodes a temperature-sensitive dominant inhibitory mutant of murine p53 (Michalovitz *et al.*, 1990); and pT22 contains an activated H-ras gene (Land *et al.*, 1983). CMV-p107 contains a full-length human p107 cDNA (Zhu *et al.*, 1993) in the cytomegalovirus enhancer/promoter-containing vector pcDNA3 (Invitrogen). P3XMyc-Elb-luc is an artificial reporter gene containing multimerized CACGTG Myc E box sequences upstream of the minimal adenovirus E1b promoter (Gupta *et al.*, 1993). GAL4-E1b-luc and GAL4-SV40-luc are GAL4 reporters which contain multimerized GAL4 sites upstream of the minimal E1b or SV40 early promoters (gifts of F Rauscher III). The ODC luciferase reporter ODCΔluc and the α-prothymosin luciferase reporters PrT-luc and GAL4mE-PrT-luc have been described (Desbarats *et al.*, 1996; Packham and Cleveland, 1997). The BacBin baculovirus was prepared by standard methods (O'Reilly *et al.*, 1992) using the baculovirus expression vector pVL1392 (Invitrogen) into which the full-

length Bin1 cDNA was subcloned. A murine c-Myc baculovirus (a gift of M Cole) was prepared similarly. GAL0 is the DNA binding domain of GAL4 (aa 1–143) and GAL4-Myc includes aa 1–262 of human Myc except the b/HLH/LZ region (Kato *et al*., 1990) which is necessary to bind Max (Prendergast *et al*., 1991). Bin1 deletion mutants and GAL4 fusion genes were subcloned for expression in pcDNA3 (the same vector as Bin1 and Bin1ΔMBD). Bin1ΔBAR-C was constructed by dropping an internal Afl III restriction fragment from CMV-Bin1, resulting in a deletion of aa 125–207 from the BAR domain (Sakamuro *et al*., 1996). The remaining mutants were generated by standard PCR methodology using the oligonucleotide primers 995′(Bam), 993′SH3(Xho) (Sakamuro *et al*., 1996) and others whose sequence is derived from the Bin1 cDNA sequence (GenBank accession number U68485). The integrity of PCR-generated fragments was verified by DNA sequencing. To conserve space, oligonucleotides and construction details are omitted but are available from GC Prendergast. Bin1ΔU1 lacks aa 224–248; Bin1ΔNLS, aa 251–269; Bin1ΔSH3, aa 384–451; the other mutants lack the residues indicated. GAL4-Bin1 fusions were generated in two steps by first subcloning the 143 aa DNA binding domain from GAL0 into pcDNA3 and then ligating in-frame full-length Bin1 or Bin1ΔMBD (lacking aa 270–383) cDNAs downstream.

Cell culture

COS, 293T, HeLa, and HepG2 cells from the ATCC were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Atlantic) and 40 U/ml penicillin and streptomycin (Fisher). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Gibco) and antibiotics (transfections were performed in media containing 10% fetal calf serum). C2C12 cells were carried in DMEM supplemented with 15% fetal bovine serum and antibiotics. Differentiation of C2C12 was induced by removing shifting cells at ~70% confluence to DMEM supplemented with 5% horse serum and antibiotics for 5 days, when myotube formation was maximal. Secondary passage rat embryo fibroblasts (REFs) were obtained from Whittaker Bioproducts and cultured and transfected as described (Prendergast *et al*., 1992). For transformation assays, secondary passage REFs seeded in 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method (Chen and Okayama, 1987) with 5 μg each of oncogenic Ras plus Myc, E1A, or mutant p53 expression plasmids and 10 μg of Bin1 plasmid or empty vector. Cells were fed and the next day passaged into one 15 cm dish (Myc transfections) or three 10 cm dishes (E1A or mutant p53 transfections). Foci were scored by methanol fixation and crystal violet staining 12–16 days later. Colony formation assays in HepG2 cells were performed by seeding ~3×10^5 cells in 6 cm dishes and transfecting the next day with 2 μg plasmid DNA using Lipofectamine (Gibco/BRL). Cells were passaged 48 h after transfection at a 1:10 ratio into 6 cm dishes containing media with ~0.6 mg/ml G418 and cell colonies were scored by crystal violet staining ~3 weeks later.

Immunoprecipitation

For insect cell experiments, ~10^7 Sf9 cells were infected with the recombinant baculoviruses indicated at an m.o.i. of approximately 10. Two days after infection, cells were harvested and ~2×10^6 cells for each IP were extracted in 0.5 ml 50 mM TrisCl pH 8/150 mM NaCl/0.1% NP40. Clarified lysates were subjected to immunoprecipitation by incubation 1.5 h at 4°C with 1 μg of anti-murine c-Myc antibody #6–213 (Upstate Biotechnology) or 100 μl hybridoma supernatant containing the Bin1 monoclonal antibody 99D (Wechsler-Reya *et al*., 1997a). Immune complexes were collected on Protein G-Sepharose (Pharmacia), washed four times with binding buffer, eluted by boiling in SDS gel loading buffer, and fractionated by SDS–PAGE. Gels were Western blotted by standard methods (Harlow and Lane, 1988) and probed with 1 μg/ml anti-Myc or a 1:50 dilution of 99D hybridoma supernatant. Blots were developed using a chemiluminescence kit (Pierce). For experiments in mouse cells, 5–10 dishes of growing or differentiated C2C12 cells were trypsinized, washed with excess growth media and then with 30 ml PBS each at 4°C. All subsequent steps were performed on ice or at 4°C. Cells were resuspended in hypotonic buffer (10 mM HEPES pH 8.0, 10 mM KCl, 0.1 mM EDTA, and 1 mM PMSF, aprotinin, leupeptin, antipain), incubated 3–5 min, and pelleted. These swollen cells were resuspended in extraction buffer (20 mM HEPES, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 0.1% NP-40, and protease inhibitors) and lysed by 10 strokes with a B pestle homogenizer. Before immunoprecipitation, the extract was incubated 15 min and clarified by a 5 min microcentrifugation. The protein concentration in the extract was determined by Bradford assay and 1.5 mg was incubated overnight with 1 μg anti-c-myc #sc-42 (Santa Cruz Biotechnology). Immune complexes were collected on protein G-agarose, washed three times with extraction buffer, and fractionated by nonreducing SDS–PAGE. Gels were Western blotted and probed as indicated with a 1:50 dilution of 99D hybridoma supernatant or ~1 μg/ml anti-Myc antibody 9E10 (Evan *et al*., 1985). Blots were developed in these experiments with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase, using an colormetric staining reaction catalyzed by this enzyme. To confirm expression of Bin1 deletion mutants, COS cells were metabolically labeled for 2 h in DMEM lacking methionine and cysteine (Gibco) with 100 μCi/ml EXPRESS labeling reagent (NEN) and cell extracts were prepared with NP40 buffer containing leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and antipain (Harlow and Lane, 1988). Extracts were microcentrifuged for 15 min at 4°C before use. Extracts were precleared by a 1 h treatment with prebleed sera or normal mouse IgG and 20 μl or a 1:1 slurry of protein G-Sepharose beads at 4°C on a nutator (Pharmacia). A mixture of hybridoma supernatants (50 μl each) containing Bin1 monoclonal antibodies 99D, 99E, and 99I were used for immunoprecipitation (Wechsler-Reya *et al*., 1997a). After incubation 1 h at 4°C, immune complexes were collected on protein G-Sepharose, washed four times with NP40 buffer, eluted in SDS gel loading buffer, fractionated on 10% SDS-PA gels, and fluorographed.

Immunofluorescence

293T were seeded onto glass cover slips in six well dishes and transfected the next day with 5 μg of the Bin1 expression vectors indicated. Two days later, cells were fixed, lysed, and processed for Bin1 immunofluorescence as described previously (Prendergast and Ziff, 1991; Sakamuro *et al*., 1996; Wechsler-Reya *et al*., 1997a), except that a 1:100 instead of a 1:5 dilution of 99D was used to limit detection to cells overexpressing the gene products of interest. Cells were photographed on a Leica immunofluorescence microscope apparatus using Ektachrome film and slides were scanned and processed with Photoshop software.

Transactivation assays

Conditions for transient Myc activation assays were taken from the reports using the various reporter genes employed (Bello-Fernandez *et al*., 1993; Desbarats *et al*., 1996; Kato *et al*., 1990; Packham and Cleveland, 1997; Zhang and Prochownik, 1997). NIH3T3 or HeLa cells were transfected using standard calcium phosphate methods and promoter sequences and total plasmid DNA in each transfection was equalized with empty vectors as appropriate. Each DNA mixture included equivalent amounts of a β-galactosidase

vector to normalize for transfection efficiency. Two days after transfection, cell extracts were prepared and analysed for luciferase and β-galactosidase activity using commercial kits, following protocols provided by the vendor (Promega).

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c-Myc mediates apoptosis through interaction with the adaptor protein Bin1

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Abstract

Bin1 is an adaptor protein and tumor suppressor that interacts with c-Myc. In this study, we demonstrate that Bin1 is necessary for c-Myc to induce apoptosis. Expression of antisense or dominant inhibitory Bin1 genes in primary chick fibroblasts had no effect on c-Myc-induced transformation but significantly reduced the susceptibility of cells to c-Myc-induced apoptosis. In particular, overexpression of the c-Myc-binding domain of Bin1 rendered cells resistant to apoptosis, implying that c-Myc-Bin1 interaction is specifically required for death and that Bin1 is part of a death effector mechanism. In baby rat kidney epithelial cells transformed by deregulated c-Myc and mutant p53 (BRK myc/p53ts cells), Bin1 inhibition promoted proliferation and blocked induction of p53-independent cell apoptosis caused by serum deprival. Colony formation assays showed that Bin1 inhibition masked the cytotoxic effects of c-Myc as potently as Bcl-2, supporting productive cell proliferation under low serum conditions. We concluded that Bin1 mediated a death or death sensitization signal from c-Myc. Our findings support the 'dual signal' model for Myc function by distinguishing its proliferative and proapoptotic activities on the basis of interactions with a binding protein. We propose that loss of Bin1 contributes to the deregulation of c-Myc in cancer cells by abolishing a mechanism which limits its ability to drive cell proliferation at inappropriate times.

Introduction

c-Myc is a nuclear oncoprotein that is necessary and sufficient to promote efficient cell cycle progression (reviewed in Henriksson and Lüscher 1996; Prendergast 1997; Facchini and Penn 1998). When deregulated by chromosomal translocation, point mutation, gene amplification, or overexpression, c-Myc contributes strongly to the malignant development of many human tumors (Cole 1986). Interestingly, under certain conditions deregulated c-Myc can also induce programmed cell death, or apoptosis (Askew *et al.* 1991; Evan *et al.* 1992). For example, following growth factor deprivation, cells that contain normal c-Myc downregulate its expression and exit the cell cycle, whereas cells that contain deregulated c-Myc maintain its expression and undergo apoptosis. The molecular mechanism(s) underlying the transforming and apoptotic properties of c-Myc have not been elucidated.

Two general models for apoptosis by c-Myc have been considered, termed the conflict and dual signal models (reviewed in Evan *et al.* 1995; Packham and Cleveland 1995). In the conflict model, apoptosis is an indirect response of the cell to an inappropriate growth signal from c-Myc. In the dual signal model, c-Myc is proposed to directly regulate growth and death pathways by interacting with effector functions specific for each process. A variant of the dual signal model proposes that c-Myc does not signal death but instead sensitizes cells to death by other agents (Evan and Littlewood 1998). In either case, the dual signal model would be favored if the proliferative and apoptotic properties of c-Myc were separable. Max interaction is necessary for both processes (Amati *et al.* 1993; Amati *et al.* 1993) but specific roles for other c-Myc-interacting proteins have not been explored.

Bin1 (Box-dependent myc-INteracting protein-1 or Bridging INtegrator-1) is a nucleocytoplasmic adaptor protein that was identified initially through its ability to interact with c-Myc (Sakamuro *et al.* 1996). While its roles as an adaptor are complex, there is significant evidence

supporting a role for Bin1 in cell growth control. Bin1 functionally associates with c-Myc in cells and selectively inhibits its oncogenic and transactivation properties (Elliott *et al.* 1999; Sakamuro *et al.* 1996). In addition, Bin1 inhibits the growth of many human tumor cell lines (DuHadaway *et al.* 1999; Elliott *et al.* 1999; Elliott *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999; Sakamuro *et al.* 1996) and similar to other tumor suppressors has been shown to be necessary for myoblast differentiation (Mao *et al.* 1999; Wechsler-Reya *et al.* 1998). Expression of the Bin1 gene (Wechsler-Reya *et al.* 1997) is missing or epigenetically altered in cancer, such as breast and prostate tumors and melanoma (DuHadaway *et al.* 1999; Elliott *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999; Wechsler-Reya *et al.* 1997). In this study, we show that c-Myc requires Bin1 to induce apoptosis and that interaction with Bin1 is implicated in mediating a p53-independent death or death sensitization signal from c-Myc. Our results support the 'dual signal' model for Myc function by showing that the transforming and apoptotic properties of c-Myc can be separated on the basis of interactions with a binding protein. We propose that Bin1 contributes to an abortive mechanism which limits the consequences of Myc deregulation in cells, one which is ablated or suppressed in neoplastic settings where Myc is deregulated.

Results

Bin1 interaction is required to mediate apoptosis by c-Myc

Previous biochemical and genetic experiments demonstrated that the c-Myc-binding domain (MBD) of Bin1 was necessary and sufficient for interaction c-Myc and that it was able to dominantly interfere with c-Myc-Bin1 interaction when overexpressed (Sakamuro *et al.* 1996). Therefore, we overexpressed the MBD as a strategy to interfere with Bin1 activity and determine whether the c-Myc-Bin1 interaction was required for c-Myc to induce apoptosis. Primary chick embryo fibroblasts (CEFs) were selected as a model system for several reasons. c-Myc deregulation is sufficient to induce both transformation and apoptosis in this model so one can examine the effects of Bin1

inhibition on each. CEFs undergo c-Myc-induced apoptosis the same way as rodent fibroblasts, displaying characteristic cell detachment, blebbing, chromatin condensation, and DNA degradation (Crouch *et al.* 1996). A major advantage of this system is the availability of replication-competent retroviral vectors (Petropoulos and Hughes 1991) which permit rapid and unselected gene transfer to large populations of cells, thereby greatly reducing selection for antiapoptotic background (a situation which develops in Myc-expressing cells after several weeks of culture). Bin1 is ubiquitously expressed in cells (Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1997). Expression of Bin1 in CEFs was confirmed with anti-Bin1 monoclonal antibody 99D (Wechsler-Reya *et al.* 1997) by Western analysis and nuclear localization was confirmed by indirect immunofluorescence (data not shown). In the initial experiments, CEFs were infected with combinations of A or B envelope subtype viruses, in which the A subtype viruses carried human c-Myc cDNA or no insert, and the B subtype viruses carried a Bin1 MBD cDNA, a human Bcl-2 cDNA (as a positive control for suppression of apoptosis by c-Myc (Bissonnette *et al.* 1992; Fanidi *et al.* 1992; Wagner *et al.* 1993; Wang *et al.* 1993), or no insert. Three days after infection, cells were harvested to verify expression of the transgenes or to test in growth and apoptosis assays.

Overexpression of the Bin1 MBD did not block the ability of c-Myc to drive CEF proliferation or to induce anchorage-independence, but rendered CEFs resistant to apoptosis by c-Myc following serum deprival almost as well as Bcl-2. Expression of transgenes in CEFs infected with A and B retroviral vectors was confirmed by Northern (data not shown) and Western analysis (see Figure 1A). Growth curves confirmed that deregulation of c-Myc promoted CEF proliferation as expected (see Figure 1B). Bcl-2 coexpression retarded the growth of c-Myc-expressing cells slightly consistent with its cell growth and cell cycle inhibitory effects (O'Reilly *et al.* 1996; Pietenpol *et al.* 1994). In contrast, MBD coexpression-expression slightly promoted proliferation in the presence of deregulated c-Myc. This effect was subtle but reproducible in different trials. To assess the effects of MBD expression on c-Myc-dependent transformation, cells were seeded into soft agar culture to assay anchorage-independent growth. All cells which expressed deregulated c-

Myc formed colonies whereas cells lacking deregulated c-Myc did not exhibit this ability (see Figure 1C). Thus, MBD did not inhibit the growth- and transformation-promoting properties of c-Myc in CEFs. To test the effects of MBD expression on apoptosis by c-Myc, we cultured cells in growth media or in low-serum media which elicits apoptosis in the presence of deregulated c-Myc. Similar to primary rodent embryo fibroblasts (Evan *et al.* 1992), and as shown by others (Crouch *et al.* 1996), CEFs overexpressing c-Myc exhibited massive signs of apoptosis within 24 hr of serum withdrawal (see Figure 2A). Strikingly, coexpression-expression of MBD suppressed apoptosis by c-Myc almost as potently as Bcl-2. The inhibitory effect was specific, because MBD did not inhibit the basal level apoptosis of cells which lacked c-Myc overexpression when they were deprived of serum. Moreover, MBD had little effect on apoptosis induced by thapsigarin (see Figure 2B), which kills through a Ca^{+2}-dependent and c-Myc-independent mechanism that is susceptible to suppression by Bcl-2 (Distelhorst and McCormick 1996). Experiments using c-Myc vectors with promoters of different strength (i.e. RCAS versus RCOS vectors (Petropoulos and Hughes 1991)) demonstrated that the inhibitory effects of MBD could be titered as c-Myc expression was driven to higher levels (data not shown). These results were consistent with the expectation that MBD acted by competing with endogenous Bin1 for interactions with c-Myc. We concluded that Bin1 interaction with c-Myc was necessary for apoptosis but not for transformation.

To corroborate these observations a set of similar experiments was performed using an antisense gene (Bin1 AS) or an inactive effector mutant that dominantly inhibited Bin1 through a different mechanism of action (Bin1Δ4). The inhibitory activity of the Bin1 AS gene against Bin1 has been documented previously in mouse cells (Wechsler-Reya *et al.* 1998). Bin1Δ4 is a deletion mutant that lacks 5 residues in the N-terminal BAR-C domain which is crucial along with the MBD for Bin1 to inhibit c-Myc/Ras cotransformation of rat embryo fibroblasts (Elliott *et al.* 1999). The Δ4 region maps to the most highly conserved part of the Bin1 gene (G.C.P., unpublished observations) and its deletion renders Bin1 inactive but capable of dominant interference with wild-type Bin1 in c-Myc/Ras cotransformation assays (see Figure 3A). Bin1Δ4 has an intact MBD and its

dominant inhibitory activity is based on interactions with proteins other than c-Myc. CEFs expressing c-Myc and Bin1 AS, Bin1Δ4, or no insert were generated by infection with recombinant retroviruses. Exogenous expression was confirmed by RT-PCR to distinguish the Δ4 transcript and (see Figure 3B) and by Northern and Western analysis (data not shown). Similar to what was seen with MBD overexpression, Bin1 AS or Bin1Δ4 did not affect the ability of c-Myc to drive CEF proliferation or transformation, as measured by growth curve determination and acquisition of anchorage-independence (see Figures 3C and 3D). In contrast, both genes significantly reduced the susceptibility to apoptosis by c-Myc elicited by serum withdrawal but not to apoptosis induced by thapsigarin (see Figure 3E). Taken together, these results supported the conclusion that Bin1 was necessary for c-Myc-induced death.

Although in previous studies we did not observe cytotoxic effects of Bin1 in untransformed cells (Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1998), since results from this study argued that Bin1 had a necessary role in apoptosis by c-Myc, we further investigated in CEFs whether Bin1 was sufficient to induce death by itself or to augment it in the presence of deregulated c-Myc. Using a recombinant retrovirus to deliver wild-type Bin1 we observed no augmentation of apoptosis when c-Myc was coexpressed, indicating that endogenous levels of Bin1 were not limiting. Furthermore, we observed that Bin1 was insufficient by itself to induce cell death in the absence of c-Myc overexpression (data not shown). These observations corroborated others indicating that overexpression of Bin1 is insufficient to induce apoptosis in the absence of c-Myc deregulation, including in primary rat embryo fibroblasts (REFs), C2C12 mouse myoblasts, IMR90 human diploid fibroblasts, or normal human melanocytes (Elliott *et al.* 1999; Ge *et al.* 1999; Sakamuro *et al.* 1996; Wechsler-Reya *et al.* 1998). Taken together, the results indicated that Bin1 had a selective and necessary role in apoptosis by c-Myc that was manifested only if c-Myc was deregulated. We concluded that Bin1 mediated apoptosis by c-Myc in a manner that was consistent with a role as a death adaptor protein.

Bin1 is required for c-Myc to induce p53-independent apoptosis in epithelial cells

We showed previously that deregulated c-Myc efficiently induces p53-independent apoptosis in baby rat kidney (BRK) epithelial cells (Sakamuro *et al.* 1995). We investigated a role for Bin1 in this process using LTR.1A, a BRK cell line developed in that study by concerted immortalization with human c-Myc and a temperature-sensitive dominant inhibitory p53 mutant. p53-independent apoptosis is induced efficiently by c-Myc in LTR.1A cells by serum deprivation at the nonpermissive temperature (38°C) for wild-type p53 function (Sakamuro *et al.* 1995). LTR.1A cells were infected by recombinant murine retroviruses expressing Bin1 AS, Bin1Δ4, or no insert, and the neomycin resistance cassette on each vector was selected for by culturing cells in G418. Drug-resistant cells were pooled and exogenous expression of Bin1 AS and Bin1Δ4 was confirmed by RT-PCR as before. No differences were observed in the number of colonies which emerged following infection nor in cell morphology, consistent with the likelihood that neither gene was growth inhibitory (data not shown). Effects on proliferation and apoptosis of BRK LTR.1A cells were assessed as before. Bin1 AS and Bin1Δ4 expression promoted the growth of LTR.1A cells relative to vector controls (see Figure 4A). The effect was similar to the trend noted in CEFs but stronger (compare Figures 1B and 3C), possibly reflecting the difference in the status of p53 in CEFs (wild-type) versus in LTR.1A cultured at 38°C (mutant). p53-independent apoptosis was induced by culturing the LTR.1A cell populations at 38°C in media containing 0.1% serum, which maintains the temperature-sensitive p53 mutant in its dominant inhibitory state (Sakamuro *et al.* 1995). Similar to the parental cell line, the vector control cells exhibited strong apoptotic death. In contrast, the cells expressing the Bin1 AS and Bin1Δ4 genes exhibited significant resistance to apoptosis (see Figures 4B and 4C). The effect of Bin1 AS and Bin1Δ4 was not due to a reduction of c-Myc levels in the BRK cells (data not shown), ruling out the trivial possibility that resistance was due to reduced levels of c-Myc that were sufficient to support immortalization but not apoptosis. We concluded that Bin1 was necessary for c-Myc to induce apoptosis in epithelial cells via a p53-independent mechanism(s).

Bin1 inhibition supports outgrowth of c-Myc-expressing cells under low serum conditions

c-Myc is sufficient to drive cell cycle progression in the absence of growth factors (Eilers *et al.* 1989). Therefore, if Bin1 is necessary for c-Myc to drive apoptosis but dispensable for it to promote cell proliferation, then inhibition of Bin1 should promote the outgrowth of c-Myc-expressing cells when they are deprived of growth factors (a situation which would normally lead to their apoptotic demise). To test this hypothesis we performed colony formation assays in Rat1A fibroblasts, which are quite sensitive to the cytotoxic effects of c-Myc. Rat1A cells were transfected with the strong human c-Myc vector LTR Hm and neomycin resistance gene-tagged vectors for Bin1 AS , Bin1Δ4, mutant p53, Bcl-2, or no insert. Rat1A fibroblasts have wild-type p53, which sensitizes them to apoptosis by c-Myc (Wagner *et al.* 1994), so coexpression-expression of mutant p53 was expected to blunt the cytotoxicity of c-Myc in these cells in a manner similar to Bcl-2 (Fanidi *et al.* 1992; Wagner *et al.* 1993). G418 selection was imposed in media containing 2% serum. This level of growth factors is insufficient to block apoptosis of rodent fibroblasts overexpressing c-Myc (Evan *et al.* 1992), so colonies would form only if cell growth could outpace cell death in the colony. After two weeks, G418-resistant colonies were stained and counted. As expected, cells transfected with LTR Hm and empty vector and cultured under these conditions formed significantly fewer colonies compared to the vector only control (see Figure 5A) The inhibitory effect of c-Myc on colony formation in 2% serum was relieved by cotransfection of Bcl-2 or also by mutant p53. Bcl-2 cooperates with c-Myc in Rat1 fibroblasts by blocking apoptosis (Fanidi *et al.* 1992; Wagner *et al.* 1993), validating this assay as a measurement of antiapoptotic potential. Notably, cotransfection of Bin1 AS or Bin1Δ4 also relieved c-Myc cytotoxicity and the relief was as potent as that provided by Bcl-2 (see Figure 5A). Experiments in which the ratio of c-Myc to Bin1 AS vector was varied in the transfected DNA demonstrated that the inhibitory effect was titratable (see Figure 5B). We noted that the effect on colony number by Bin1 AS or Bin1Δ4 was similar but that Bin1 AS had a more pronounced effect on colony size (see Figure 5C), indicating its action was slightly stronger. The results corroborated findings in CEFs and BRKs

which indicated that inhibition of Bin1 selectively suppressed apoptosis but not proliferation by c-Myc. We concluded that Bin1 mediated the cytotoxicity of c-Myc and thereby limited the ability of c-Myc to drive cell proliferation under low serum conditions.

Discussion

This study offers evidence of a selective and necessary role for the c-Myc binding adaptor protein Bin1 in activation of apoptosis by c-Myc. This role was manifested in fibroblasts, where p53 status has been argued to be important (Hermeking and Eick 1994; Wagner *et al.* 1994), and in epithelial cells, where it is not (Sakamuro *et al.* 1995). Notably, the c-Myc binding domain (MBD) of Bin1 was a potent and specific inhibitor of cell death. Since this domain is necessary and sufficient for interaction with c-Myc (Elliott *et al.* 1999; Sakamuro *et al.* 1996), its antiapoptotic action implied that interaction between Bin1 and c-Myc is necessary for death and that Bin1 is a specific death effector or coactivator which acts downstream or in parallel to c-Myc, respectively. Bin1 was not sufficient to induce death when overexpressed in the absence of deregulated c-Myc. Thus, Bin1 is not an 'executioner' but an adaptor protein that under certain circumstances can link c-Myc to a bona fide death pathway. In growing cells, c-Myc and Bin1 are coexpressed, colocalized, and can be coimmunoprecipitated (Elliott *et al.* 1999; Wechsler-Reya *et al.* 1997; Wechsler-Reya *et al.* 1998), other events or conditions must potentiate the death signal which Bin1 is needed to mediate. Since c-Myc and Bin1 are each phosphoproteins and c-Myc is known to be regulated by phosphorylation (Lutterbach and Hann 1994; Wechsler-Reya *et al.* 1997), it is tempting to speculate that the activity of each protein is regulated by kinases that are in turn regulated by survival factors. Pathways regulated by insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) are intriguing in this regard since both factors have been shown to be sufficient to suppress apoptosis by c-Myc in fibroblasts (Harrington *et al.* 1994). This study prompts investigation of the role of phosphorylation in regulating proapoptotic functions of Bin1.

We observed that Bin1 was necessary for apoptosis by c-Myc in BRK epithelial cells where p53 is dispensable. Thus, Bin1 may act in parallel or downstream of p53, providing a mechanism to explain how c-Myc can kill in certain cell types when p53 is abolished by mutation. The exact relationship between c-Myc and p53 in apoptosis is uncertain. While several studies performed in fibroblast and lymphocyte models suggest p53 is required (Hermeking and Eick 1994; Lotem and Sachs 1995; Wagner *et al.* 1994; Wang *et al.* 1993), and suggest a linkage by $p19^{ARF}$ (Zindy *et al.* 1998), other studies performed in quite similar models argue strongly against a role for p53 (Hsu *et al.* 1995; Lenahan and Ozer 1996). p53 is clearly not required in some cell types, such as BRK epithelial cells, where c-Myc can promote apoptosis by p53-dependent or p53-independent mechanisms (Sakamuro *et al.* 1995). Tissue-specific cooperation of p53 and c-Myc in apoptosis is suggested by the observation that c-Myc activation and p53 inactivation cooperate to promote thymic lymphoma but not mammary carcinoma (Elson *et al.* 1995). A recent study of the p53 modulator $p33^{ING1}$ in cells where apoptosis is induced by deregulated c-Myc is compatible with the notion that the death pathways activated by p53 and c-Myc are arranged in a parallel rather than strictly epistatic configuration (Helbing *et al.* 1997). One way to consider the relationship between p53 and c-Myc is to propose that each sensitize cells to apoptosis determined by each other or to other factors, such as, for example, DNA damage in the case of p53 (Lane 1992), or Fas or TNF in the case of c-Myc (Hueber *et al.* 1997; Klefstrom *et al.* 1997). In this scenario, Bin1 might mediate a death sensitization signal from c-Myc that could act independently but also cooperatively with p53 elevation. Indeed, a general role for Bin1 in sensitizing cells to apoptotic signals should be entertained, because the Bin1 gene appears to be a transcriptional target of the apoptosis-regulating factor NF-κB (Mao *et al.* 1999). It will be interesting to investigate links between Myc, Bin1, and p53 in BRK epithelial cells which exhibit two modes of apoptosis by c-Myc (Sakamuro *et al.* 1997), and to determine whether Bin1 is necessary for Myc and/or p53 to sensitize cells to apoptosis by stimuli other than growth factor deprival.

Our findings support the 'dual signal' model which proposes that regulation of apoptosis is a physiological component of c-Myc action (Harrington *et al.* 1994; Harrington *et al.* 1994). Others have shown that dibutyryl cAMP can arrest cells containing deregulated c-Myc without affecting their sensitivity to apoptosis by growth factor deprival, consistent with the notion that different effectors of Myc mediate its different biological effects (Packham and Cleveland 1996). Thus, c-Myc may induce cell death via a novel effector pathway involving Bin1 and induce cell proliferation and transformation through other c-Myc binding proteins, such as, for example, the recently described ATM-related protein TRRAP, which is crucial for c-Myc-dependent cell transformation (McMahon *et al.* 1998). The 'dual signal' model shown in Figure 6 incorporates Bin1 into a p53-independent apoptosis pathway. As mentioned above, Bin1 is a phosphoprotein and therefore conceivably a target for regulation by growth factors that modulate apoptosis by c-Myc (Harrington *et al.* 1994). This model does not distinguish a specific role for Bin1 in cases where c-Myc is overexpressed rather than merely deregulated, an issue that may contribute to whether p53-dependent or p53-independent mechanisms for apoptosis may be favored in cells. However, at this time we do not favor such a distinction, because the potency of the apoptotic activity of Bin1 in tumor cells does not correlate with their degree of c-Myc overexpression (K. Ge, K. Elliott, and G.C.P., unpublished observations). Interestingly, recent experiments indicate that Bin1 induces tumor cell death by a caspase-independent mechanism associated with membrane blebbing (Elliott *et al.* 1999), reminiscent of the features of cell death induced by c-Myc in the presence of caspase inhibitors (McCarthy *et al.* 1997). Thus, Bin1 may address the gap in knowledge concerning how c-Myc triggers commitment to death independently of caspases and at a point before caspases come into play (McCarthy *et al.* 1997).

Although its exact function remains obscure, c-Myc has been implicated in transcriptional regulation and ~30 genes regulated by c-Myc have been identified (Dang 1999; Facchini and Penn 1998; Henriksson and Lüscher 1996; Prendergast 1997). However, we did not detect any changes in the expression of several target genes implicated in apoptosis by Myc, including ornithine

decarboxylase, CDC25A, or Fas ligand (Galaktionov *et al.* 1996; Hueber *et al.* 1997; Packham and Cleveland 1994), associated with altered apoptotic responses in CEFs or BRKs (unpublished results). These observations are consistent with the lack of an unambiguous role for any of the c-Myc target genes identified to date in apoptosis (Dang 1999; Evan and Littlewood 1998). In transient assays, Bin1 can specifically suppress Myc transactivation (Elliott *et al.* 1999), so if this activity is physiologically germane, then other target genes may be important. Conversely, if the effects of Bin1 on transactivation are an epiphenomenon of protein-protein association as scored in transient assays, then Bin1 may have a signaling role that is independent of transactivation. The latter possibility needs to be entertained because not all biological actions of c-Myc can be ascribed strictly to gene transactivation (Gusse *et al.* 1989; Lemaitre *et al.* 1995; Li *et al.* 1994; Prendergast and Cole 1989; Yang *et al.* 1991) and Bin1 has features of a signaling protein (e.g. it has an SH3 domain). Moreover, c-Myc-induced death appears to be separable into "priming" and "initiation" steps, in which the former is associated with gene regulation but the latter is not, based on the ability of c-Myc to trigger cell death when protein synthesis is inhibited (Wagner *et al.* 1994). Identification of Bin1 effectors in cell death are needed to unravel its relationship with transcription by c-Myc, if any.

The effector functions of Bin1 are clearly of interest but currently undefined. Bin1 is in excess to Myc in cells and it has Myc-independent roles in cell regulation (Elliott *et al.* 1999). Thus, like most adaptor proteins, Bin1 probably participates in diverse interactions in the cell. Current studies of Bin1 support some role in coordinating cell fate decisions that are made when cells exit the cell cycle (e.g. arrest in G0, commit to differentiate, undergo apoptosis, etc.). For example, as shown above, if cells can not exit the cell cycle due to c-Myc deregulation, then Bin1 is necessary to mediate an abortive apoptotic signal. Alternately, if c-Myc is downregulated appropriately and as a result cells can exit the cell cycle, then Bin1 promotes cell differentiation (Wechsler-Reya *et al.* 1998). Additional information indicates that the function of Bin1 is complex. Bin1 is subjected to complex patterns of alternate splicing, especially in neurons (Butler *et al.* 1997; Ramjaun and

McPherson 1998; Ramjaun *et al.* 1997; Tsutsui *et al.* 1997; Wechsler-Reya *et al.* 1997), and it is localized to the cytosol as well as the nucleus in certain cells (Butler *et al.* 1997; Kadlec and Pendergast 1997; Wechsler-Reya *et al.* 1998). The terminal regions of Bin1 are structurally similar to amphiphysin, a neuron-specific protein and paraneoplastic autoimmune antigen in breast and lung cancer (David *et al.* 1994; Dropcho 1996), and to RVS167 and RVS161, two negative regulators of the cell cycle in yeast (Bauer *et al.* 1993; Crouzet *et al.* 1991). Amphiphysin and brain-specific splice forms of Bin1, also termed amphiphysin II or amphiphysin isoform, have been implicated in receptor-mediated endocytosis (David *et al.* 1996; Owen *et al.* 1998; Wigge *et al.* 1997). RVS167 and RVS161 have been implicated in endocytosis and karyogamy (Brizzio *et al.* 1998; Munn *et al.* 1995). Nonneuronal splice forms of Bin1 are unlikely to be involved in endocytosis, however, because only neuronal splice forms include exons which encode clathrin-binding determinants needed for localization to endocytotic vesicles (Ramjaun and McPherson 1998). It is tempting to speculate that the endocytosis connection in neurons reflects the link in those cells between survival and the achievement of a differentiated and synaptically active state associated with neurotransmitter release and hence membrane trafficking. Recently, the nuclear tyrosine kinase c-Abl was shown to associate with but not to phosphorylate Bin1 in cells (Kadlec and Pendergast 1997). Association with c-Abl is mediated by the SH3 domain in Bin1, which is dispensable for association with c-Myc (Elliott *et al.* 1999; Sakamuro *et al.* 1996). How Bin1 influences the complex actions of c-Abl in cell growth, differentiation, and apoptosis remains to be determined. However, in light of c-Abl interaction, it is interesting to note that a fraction of c-Abl in cells has been reported to be activated by localization to focal adhesions (Lewis *et al.* 1996; Taagepera *et al.* 1998), and that apoptosis by c-Myc is suppressed by signaling from integrins (Crouch *et al.* 1996), which localize to focal adhesions. Thus, there may be a link between apoptosis by c-Myc and Bin1, integrin signaling, and activation of c-Abl. In future work, it will be important to determine whether any of the existing interactions mediate Bin1 actions in cell death or differentiation or are instead regulatory in nature.

Loss of Bin1 in cancer cells may promote c-Myc deregulation by eliminating an abortive apoptotic signaling mechanism that limits the consequences of inappropriate c-Myc expression. Loss or alteration of a proapoptotic adaptor such as Bin1 would achieve this end and leave proliferation intact. Apoptosis is crucial to stanch inappropriate cell proliferation but apoptotic mechanisms are progressively eliminated during neoplastic progression (Williams 1991). In certain cancers, such as prostate and breast cancer (Kyprianou *et al.* 1991; Kyprianou *et al.* 1990; McDonnell *et al.* 1992), there is evidence that loss of such mechanisms correlates with malignant conversion. p53 mutation is probably important but other yet undefined events also seem likely to be crucial. For example, as mentioned above, while p53 null mice are more susceptible to c-Myc-induced thymic lymphoma they have the same susceptible as wild-type mice to c-Myc-induced mammary carcinoma (Elson *et al.* 1995). Bin1 has features of a tumor suppressor that is epigenetically altered or eliminated in certain cancers, including prostate cancer, breast cancer, and melanoma (DuHadaway *et al.* 1999; Ge *et al.* 1999; Ge *et al.* 1999), where at later stages c-Myc is often overexpressed and associated with poor prognosis (Berns *et al.* 1992; Borg *et al.* 1992; Hehir *et al.* 1993; Jenkins *et al.* 1997). It will be important to investigate the effects of Bin1 loss in animal tumor models where its potential contribution to c-Myc deregulation and malignant development can be fully assessed.

Materials and Methods

Plasmid and retrovirus construction. The expression vectors used in this study have been described. pcDSRα is a strong mammalian vector which uses a hybrid SV40 early region/HTLV1 U5 LTR enhancer promoter to drive expression (a gift of A. Noda). MSCVneoEB is a murine MLV retroviral vector that includes a neomycin resistance gene cassette (Hawley *et al.* 1994). RCAS (BP) (A and B envelope subtypes) and RCOS (BP) (A envelope subtype) are related chicken retroviral vectors that are competent for replication (kindly provided by S. Hughes). RCAS (BP) contains the avian leukemia virus long terminal repeat (LTR) which is about ~10-fold more active than the RAV-0 LTR in RCOS (Petropoulos and Hughes 1991). A C-terminal fragment of murine Bin1 encompassing the Myc-binding domain (MBD) which was engineered for expression by the addition of a Kozak translation initiation sequence, termed ATG99, has been described previously (Sakamuro *et al.* 1996). ATG99 is sufficient for interaction with c-Myc and dominantly interferes with c-Myc-Bin1 interaction *in vivo* (Sakamuro *et al.* 1996). ATG99 was subcloned into RCAS (BP)-B to generate RCAS-MBD. The dominant inhibitory mutant Bin1Δ4 was generated by standard PCR methodology as part of a structure-function analysis to identify crucial regulatory regions of Bin1 (Elliott *et al.* 1999). Bin1Δ4 lacks aa 143-148 of full length human Bin1 (Sakamuro *et al.* 1996). Antisense Bin1 and Bin1Δ4 cDNAs were subcloned for mammalian cell expression into pcDSRα and MSCVneoEB, generating SRα-Bin1 AS and SRα-Bin1Δ4 or MSCV-Bin1 AS and MSCV-Bin1Δ4. For chick expression the same cDNAs were subcloned into RCAS (BP)-B, generating RCAS-Bin1 AS or RCAS-Bin1Δ4, via the adaptor cloning plasmid pCla12-Nco (Hughes *et al.* 1987). A human c-Myc cDNA derived from CMV Hm (Prendergast *et al.* 1991) was subcloned into RCAS (BP)-A or RCOS (BP)-A to generate RCAS-c-Myc or RCOS-c-Myc. An RCAS-Bcl-2 vector has been described (Givol *et al.* 1994). For rat embryo fibroblast (REF) transformation experiments, Bin1Δ4 was subcloned into the cytomegalovirus (CMV) enhancer/promoter-driven vector pcDNA3 (Invitrogen), generating CMV-Bin1Δ4, to permit comparison of activity with CMV-Bin1 (Sakamuro *et al.* 1996). Similar CMV vectors for the murine temperature-sensitive dominant inhibitory mutant

p53 and human Bcl-2 have been described (Sakamuro *et al.* 1995). The activated H-ras vector pT22 and the human c-Myc vector LTR Hm used for REF focus assays also have been described (Kelekar and Cole 1986; Land *et al.* 1983).

Tissue culture and cell line generation. All cells were cultured in Dulbecco's modified Eagle media (DMEM) containing 10% fetal calf serum (Life Technologies) and penicillin/streptomycin unless otherwise indicated. Primary chick embryo fibroblasts (CEFs) were cultured from 10 day old chick embryos (EV-0 strain) by standard technique and grown in DMEM supplemented with 8% fetal calf serum, 2% chicken serum, 10% Tryptose Phosphate Broth, and antibiotics. Low passage CEFs seeded into 100 mm dishes were transfected with 5 μg each of the desired recombinant retroviral vector DNAs by a standard calcium phosphate coprecipitation method (Chen and Okayama 1987). Cells were cultured 4-7 days and passaged every other day to promote propagation of the recombinant viruses. Complete propagation was confirmed by immunofluorescence staining with an antibody to the viral core protein p27 (SPAFAS, Inc.) and culture supernatant was harvested and stored at -80°C. Viral titers achieved in this manner were typically ~10^6/ml. To generate CEF populations carrying two transgenes, cells were transfected with A subtype viral vectors, cultured 4 days, and then infected with B subtype viruses generated as above. Growth curves were performed by seeding 2 x 10^5 cells into 6 cm dishes and counting viable cells at various times later. Soft agar culture to assay anchorage-independent growth was performed by seeding 2.5 x 10^4 cells per well in triplicate into 6 well dishes, as described previously (Press *et al.* 1992), except that concanavalin A was omitted. Colonies were documented by photography at 40x magnification using an Olympus inverted microscope with a 35 mm camera attachment.

The BRK myc/p53ts cell line LTR.1A has been described previously (Sakamuro *et al.* 1995). LTR.1A cells were infected with MSCV retroviruses harvested 48 hr after transient transfection of 293-BOSC cells as described (Pear *et al.* 1993). Cells stably integrating the vector were selected in growth media containing 0.5 mg/ml G418 and pooled. Primary rat embryo fibroblasts (REFs)

(Whittaker Bioproducts) were cultured and transfected as described (Prendergast *et al.* 1992; Prendergast *et al.* 1991). Transformed foci were scored 12-16 days posttransfection. Rat1A cells (a gift of N. Kohl) were used for colony formation assays as follows. ~3 x 10^5 cells were seeded per well in 6-well dishes and transfected the next day by a standard calcium phosphate coprecipitation method (Chen and Okayama 1987) with 6 μg LTR Hm and 6 μg of empty vector, pSRα-Bin1 AS, pSRα-Bin1Δ4, CMV-p53ts, or CMV-Bcl-2 (Sakamuro *et al.* 1995). Cells were trypsinized 48 hr after transfection and seeded at a 1:20 ratio into 10 cm dishes containing DMEM media supplemented with 2% fetal calf serum and 0.6 mg/mL G418 (Life Technologies). G418-resistant colonies were scored by crystal violet staining 14 days later.

RNA analysis. The human Bin1 specific primers Δ4-sense (5'-AGT TCC CCG ACA TCA AGT CAC GCA-3') and Δ4-antisense (5'-CTT GGC AAT TTT GGC TTC ATC C-3'), which span the 18 nt deletion in Bin1Δ4, were used to document exogenous expression of Bin1Δ4 message in cells. Reverse Transcriptase (RT)-PCR analysis was performed as follows. Two μg total cytoplasmic RNA was mixed with 50 pmol of each primer, heated to 70°C for 5 min, and cooled rapidly on wet ice. RT reaction was performed in 30 μl as suggested by the Mo-MLV RT vendor (Life Technologies). Ten percent of the reaction product was used as a template for 30 cycles of PCR (denaturation 30s at 94 °C/annealing 45 s at 55 °C/polymerization 60s at 72 °C). Ten percent of the PCR product was examined by agarose gel electrophoresis and photographed. For Northern analysis, 20 μg total cytoplasmic RNA per lane was analyzed essentially as described (Prendergast and Cole 1989). Blots were probed with cDNAs for murine ODC (a gift of J. Cleveland), murine CDC25A (a gift of D. Beach), murine Fas and Fas ligand (gifts of S. Nagata), or p16INK4-exon 1β specific for $p19^{ARF}$ (a gift of T. Kamijo).

Protein analysis. For Western blotting, cell lysates was prepared in NP40 buffer (Bin1 and Bcl-2) or RIPA buffer (c-Myc) using standard protocols (Harlow and Lane 1988). For c-Myc analysis, 1.5 mg cell lysate was subjected to immunoprecipitation with 1 μg anti-c-Myc antibody

SC-42 (Santa Cruz Biotechnology) and 25 µl protein G-agarose (Life Technologies) and immunoprecipitates were washed 3 times with RIPA buffer before fractionation by non-reducing SDS-PAGE. For Bin1 and Bcl-2 analysis, 50 µg cell lysate was fractionated directly by reducing SDS-PAGE. Gels were electrophoretically transferred to ECL membrane (Amersham) or Immobilon-P (Millipore) using standard methods (Harlow and Lane 1988). Blots were blocked in 3% skim milk and probed with the anti-Bin1 monoclonal antibody 99D (Wechsler-Reya *et al.* 1997), anti-c-Myc antibody 9E10 (Evan *et al.* 1985), or anti-Bcl-2 antibody #124 (DAKO). Antibodies were diluted 1:50 in PBS with 2.5% skim milk and 0.1% Triton X-100 and incubated with the membrane 12 hr at 4°C. Blots were washed and incubated 1 hr in the same buffer with secondary goat anti-mouse antibodies conjugated to horseradish peroxidase (BMB) and developed using a chemiluminescence kit using the protocol suggested by the vendor (Pierce). Indirect immunofluorescence of BRK cells and CEFs was performed with anti-Bin1 99D as described (Prendergast and Ziff 1991; Sakamuro *et al.* 1996).

Apoptosis assays. ~10^6 CEFs expressing the transgenes indicated were seeded overnight into 60 mm dishes in complete growth media for apoptosis assays. Recombinant retrovirus-infected BRK/myc/p53ts cells were grown to ~70% confluence in 100 mm dishes. At the start of the experiment, cells were washed twice with PBS and then placed in DMEM containing either 10%, 0.1%, or 0.05% fetal calf serum as indicated. Thapsigarin (Calbiochem) was added to complete growth media where indicated to a final concentration of 100 nM as a means to induce apoptosis by a c-Myc-independent mechanism. At the end of the incubation period, cells were trypsinized, washed once with PBS, fixed and stained with propidium iodide, and processed for flow cytometry (Givol *et al.* 1994; Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). Alternately, cells were harvested, stained with acridine orange (Sigma), and subjected to fluorescence microscopy to monitor for chromatin condensation. Staining with acridine orange was performed by dissolving 2.5 mg in 50 mL of PBS at room temperature, mixing 2 µl with 10 µl of cell suspension on a slide glass, and covering with melted paraffin wax.

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Figure Legends

Figure 1. MBD overexpression does not inhibit proliferation or cell transformation by c-Myc. **(A).** Transgene expression. Cell extracts were prepared from CEFs infected with RCOS (A) vector or RCOS-c-myc plus RCAS (B) vector, RCAS-MBD, or RCAS-Bcl-2 and subjected to Western analysis with anti-c-Myc, anti-Bin1, or anti-Bcl2 antibodies. **(B.)** Anchorage-dependent growth. $2x10^5$ CEFs expressing the genes indicated were seeded into 60 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(C.)** Anchorage-independent growth. 2.5×10^4 CEFs were seeded into semisolid growth media in 6 well dishes as described in the Materials and Methods. Representative fields were documented by photomicrography at 40x magnification 10 days later.

Figure 2. MBD overexpression inhibits apoptosis by c-Myc. (A.) Apoptosis following serum deprival. CEFs expressing the transgenes indicated were incubated in growth media or in DMEM containing 0.05% fetal calf serum for 28 hr, trypsinized, fixed and stained with propidium iodide, and subjected to flow cytometry (Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). The proportion of cells in each population exhibiting sub-G1 phase DNA is displayed on the X-axis. The results of three trials are shown **(B.)** Apoptosis following thapsigarin treatment. CEFs expressing the transgenes indicated were incubated in growth media containing 100 nM thapsigarin for 24 hr and processed as above for flow cytometry. The proportion of cells in each population exhibiting sub-G1 phase DNA is displayed on the X-axis. Cells also exhibited morphological features of apoptosis and chromatin collapse (data not shown). The results of three trials are shown.

Figure 3. Inhibition of c-Myc-induced apoptosis, but not transformation or proliferation, by antisense Bin1 or the dominant inhibitory effector mutant Bin1Δ4 . (A.) Bin1Δ4 is an effector mutant with dominant inhibitory activity. Bin1Δ4 lacks the perfectly conserved aa 143-148 within the BAR-C region of Bin1 which is crucial in addition to the MBD to inhibit c-Myc transforming

activity (Elliott *et al.* 1999). REF cotransformation assays were performed using c-Myc and oncogenic H-Ras vectors LTR Hm and pT22 as described previously (Prendergast *et al.* 1992). Briefly, cells were transfected with 5 μg each oncogene plus 5 μg of CMV vector and CMV-Bin1, CMV-Bin1Δ4, or CMV vector, or plus 5 μg each of CMV-Bin1 and CMV-Bin1Δ4. Foci were scored 2 weeks later and plotted as proportion of the foci scored in the presence of CMV vector alone. The results of 5 trials are shown. Coexpression of Bin1Δ4 and Bin1 in the foci pooled from one trial in which both genes were transfected was confirmed by RT-PCR (as described in the Materials and Methods), demonstrating that Bin1 could be expressed in cells transformed by c-Myc if Bin1Δ4 was coexpressed. **(B).** Transgene expression in CEFs. Bin1 activity is fouled by the addition of either C- or N-epitope tags (Elliott *et al.* 1999; Sakamuro *et al.* 1996) so expression of the untagged Bin1Δ4 gene was confirmed by RT-PCR analysis of total cytoplasmic RNA isolated from each cell population as described in the Materials and Methods. Primers were specific for human Bin1 and do not crosshybridize to chicken Bin1 sequences. CMV-Bin1 and CMV-Bin1Δ4 were used as positive control templates. Western analysis confirmed expression of Bin1Δ4 protein and indicated truncation to two ~40 kD fragments during preparation of cell lysates. Immunoprecipitation of endogenous chicken Bin1 from CEFs that were infected with Bin1 AS retrovirus and metabolically labeled with ^{35}S-methionine indicated a ~2-fold reduction in protein levels, a degree of suppression that was similar to that associated with biological effect by the same gene on Bin1 function in mouse C2C12 cells (Wechsler-Reya *et al.* 1998). **(C.)** Anchorage-dependent growth. $2x10^5$ CEFs expressing the genes indicated were seeded into 60 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(D.)** Anchorage-independent growth. 2.5×10^4 CEFs expressing the transgenes indicated were seeded into semisolid growth media in 6 well dishes as described in the Materials and Methods. Representative fields were documented by photomicrography at 40x magnification 10 days later. **(E.)** Apoptosis following serum deprivation or thapsigarin treatment. CEFs expressing the transgenes indicated were incubated ~22 hr in growth media, DMEM containing 0.1% fetal calf serum, or growth media containing 100 nM thapsigarin. At the end of this period cells were

harvested, fixed and stained with acridine orange or propidium iodide, and subjected to flow cytometry or to fluorescence microscopy (Sakamuro *et al.* 1995; Sakamuro *et al.* 1997). The graph shows the proportion of cells in each population which exhibited apoptosis and chromatin condensation. Relative cell death as measured by the proportion of sub-G1 phase cells exhibited a slightly more pronounced suppression by Bin1 AS and Bin1Δ4 than that determined by the chromatin condensation method shown (data not shown). The results of four trials are shown.

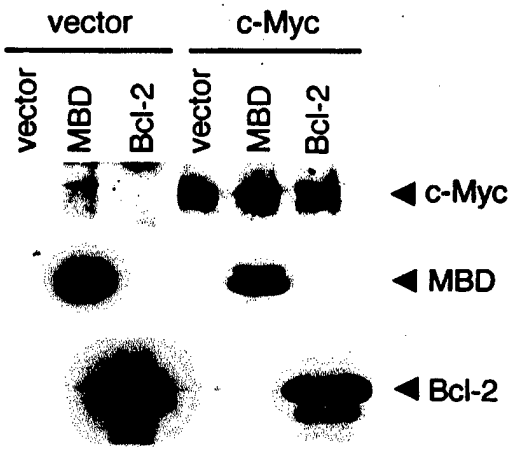
Figure 4. Bin1 is necessary for p53-independent apoptosis by c-Myc in BRK epithelial cells. **(A.)** Growth curve. 2 x 10^5 BRK LTR.1A cells expressing the genes indicated were seeded into 100 mm dishes on day 0. Viable cell counts were determined at the times indicated and cell number was graphed as a function of time. **(B.)** Response to serum deprival in the presence of p53 mutant. BRK LTR.1A cells were subjected to serum deprival as described previously (Sakamuro *et al.* 1995) and processed for flow cytometry. The relative proportion of cells exhibiting sub-G1 phase DNA is depicted on the X-axis of the graph. The results of three trials are shown. Similar suppression by Bin1 AS and Bin1Δ4 was observed in the BRK myc/p53ts cell line LTR.8C (Sakamuro *et al.* 1995) (data not shown). **(C.)** Photomicrographs of BRK LTR.1A at end of period of serum deprival.

Figure 5. Inhibition of Bin1 abolishes the cytotoxicity of c-Myc and promotes its ability to drive proliferation under low serum conditions. (A.). Relief of c-Myc cytotoxicity. Rat1A fibroblasts were transfected with 20 μg of LTR Hm or empty vector (to control for the promoter in LTR Hm) plus 5 μg of CMV vectors for the genes indicated. The CMV vector used, pcDNA3-neo, contains a neomycin-resistance cassette to permit G618 selection. G418-resistant colonies were scored 12-14 days after transfection. The results of three trials are shown. **(B.)** Titration of c-Myc cytotoxicity by Bin1 AS. Rat1A fibroblasts were transfected with 5 μg CMV-Bin1 AS or empty CMV vector (pcDNA3-neo) plus the amount of LTR Hm shown. Empty vector for the latter was used to control for the promoter in LTR Hm and to maintain plasmid amount to 20 μg in each transfection. G418-resistant colonies were scored as before. The results of three trials are shown.

(C.) Representative dishes from one experiment are shown illustrating relief by Bin1 AS and Bin1Δ4 and slight difference on colony size.

Figure 6. Model. In the 'dual signal' model for Myc function, Bin1 is proposed to have a specific adaptor role in mediating a death or death sensitization signal from c-Myc. This signal is p53-independent so p53 may be involved in parallel or upstream of Bin1. Growth factors that suppress c-Myc-induced apoptosis may target Bin1 for inactivation, perhaps by phosphorylation. Bin1 is dispensable for c-Myc to drive proliferation or transformation, which may be mediated by other proteins that interact with the c-Myc N-terminus, such as TRRAP (McMahon *et al.* 1998).

A)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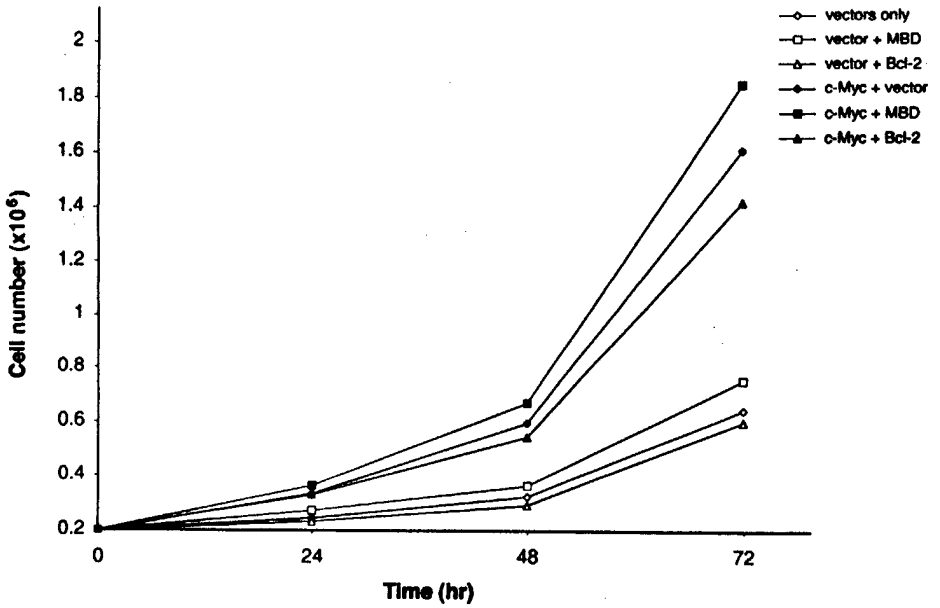
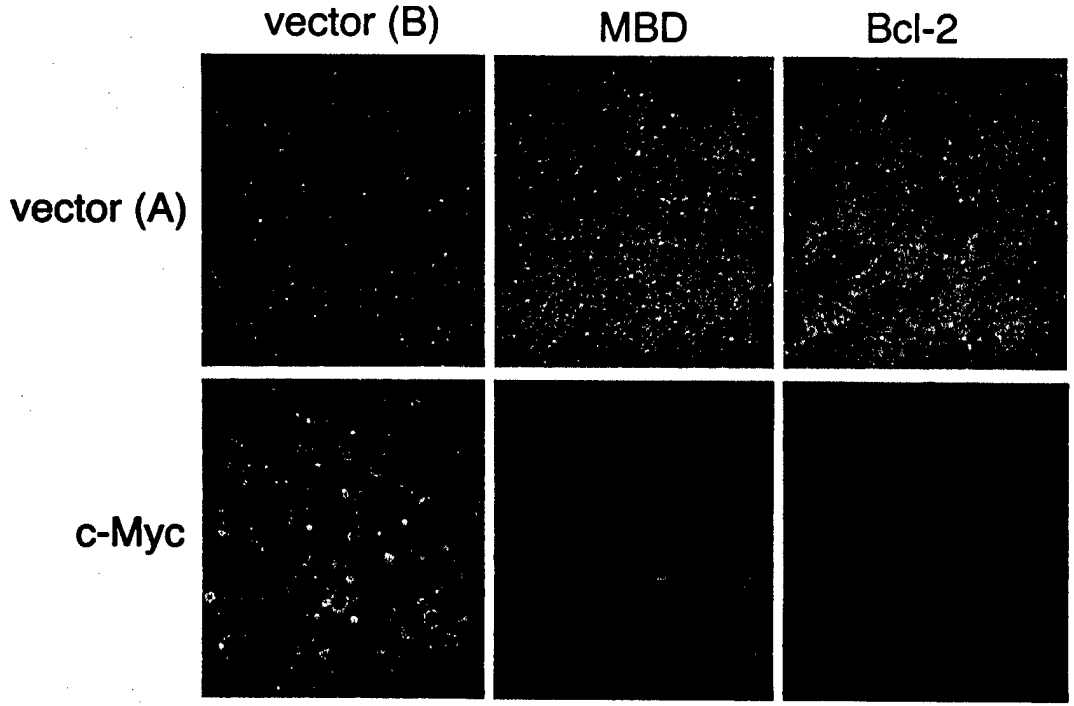
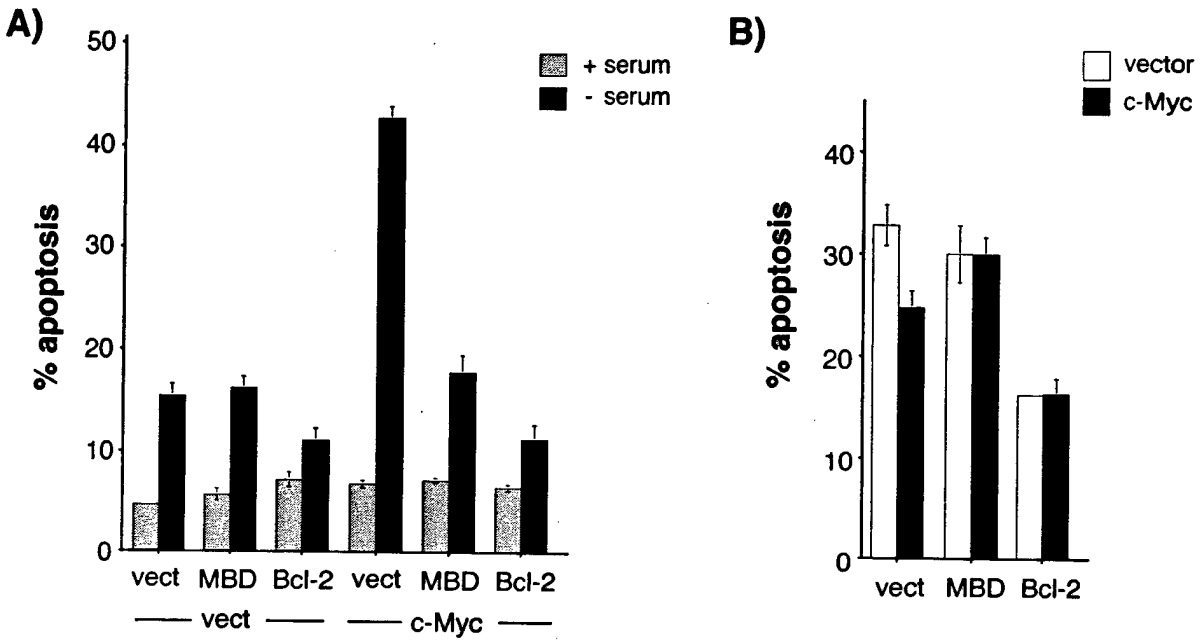
c-Myc
vector
MBD
Bcl-2
vector
MBD
Bcl-2
c-Myc
MBD
Bcl-2

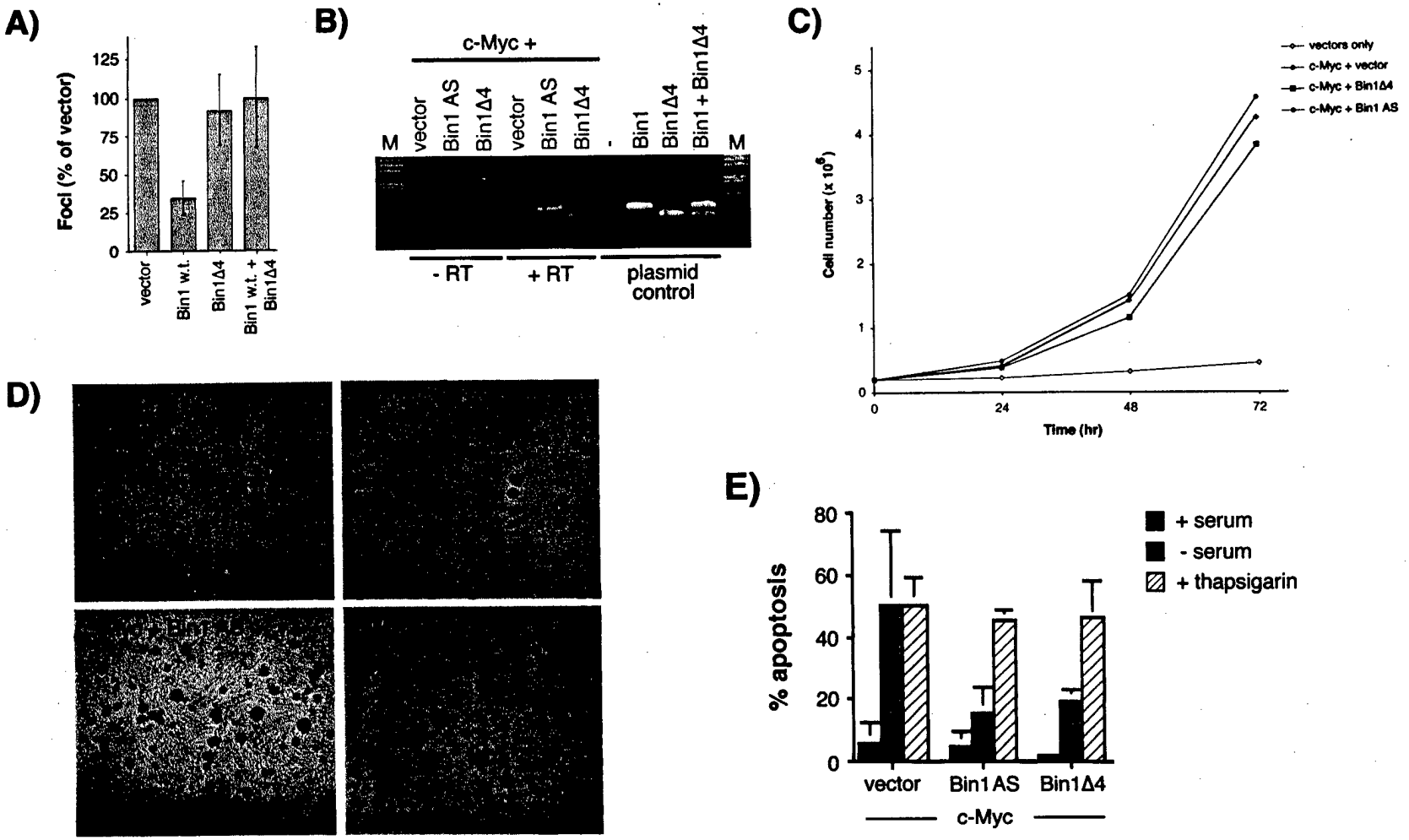
B)


vectors only
vector + MBD
vector + Bcl-2
c-Myc + vector
c-Myc + MBD
c-Myc + Bcl-2
Cell number (x10⁶)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24
48
72
Time (hr)

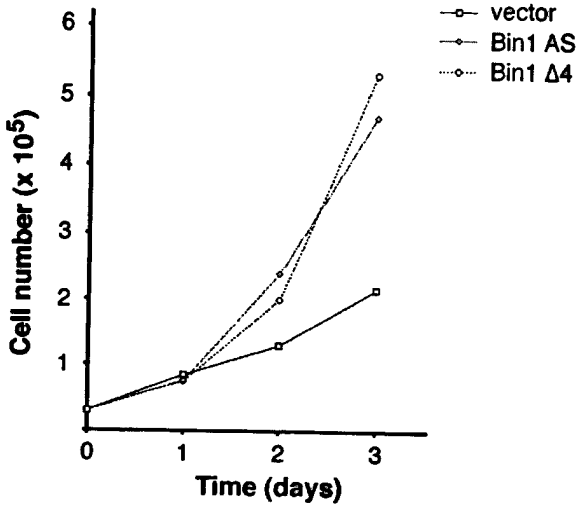
C)


vector (B)
MBD
Bcl-2
vector (A)
c-Myc

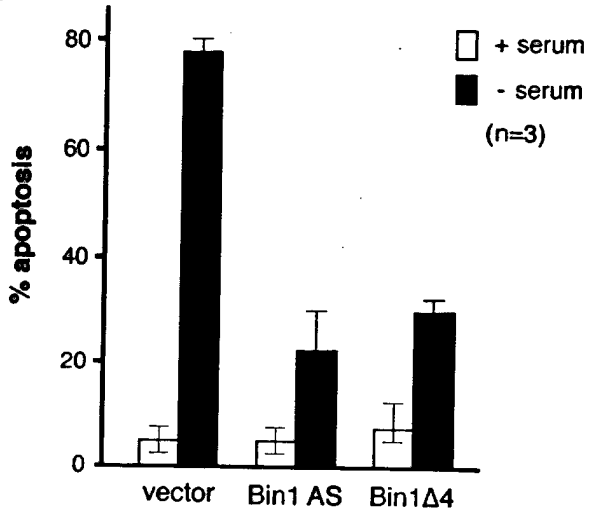

A)
50
40
30
20
10
0
% apoptosis
+ serum
- serum
vect
MBD
Bcl-2
vect
MBD
Bcl-2
vect
c-Myc
B)
vector
c-Myc
40
30
20
10
0
% apoptosis
vect
MBD
Bcl-2


A)
Foci (% of vector)
125
100
75
50
25
0
vector
Bin1 w.t.
Bin1Δ4
Bin1 w.t. + Bin1Δ4
B)
c-Myc +
M
vector
Bin1 AS
Bin1Δ4
vector
Bin1 AS
Bin1Δ4
-
Bin1
Bin1Δ4
Bin1 + Bin1Δ4
M
- RT
+ RT
plasmid control
C)
vectors only
c-Myc + vector
c-Myc + Bin1Δ4
c-Myc + Bin1 AS
Cell number (x 10^6)
5
4
3
2
1
0
0
24
48
72
Time (hr)
D)
E)
% apoptosis
80
60
40
20
0
+ serum
- serum
+ thapsigarin
vector
Bin1 AS
Bin1Δ4
c-Myc

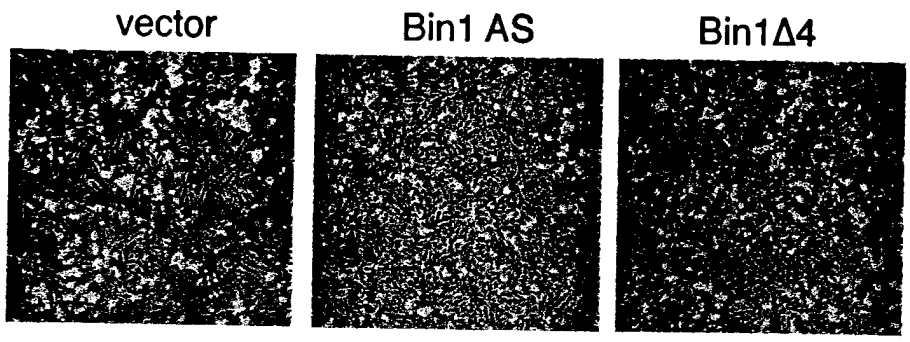
A)


vector
Bin1 AS
Bin1 Δ4
Cell number (x 10^5)
Time (days)
0
1
2
3
4
5
6

B)


+ serum
- serum
(n=3)
% apoptosis
0
20
40
60
80
vector
Bin1 AS
Bin1Δ4

C)


vector
Bin1 AS
Bin1Δ4

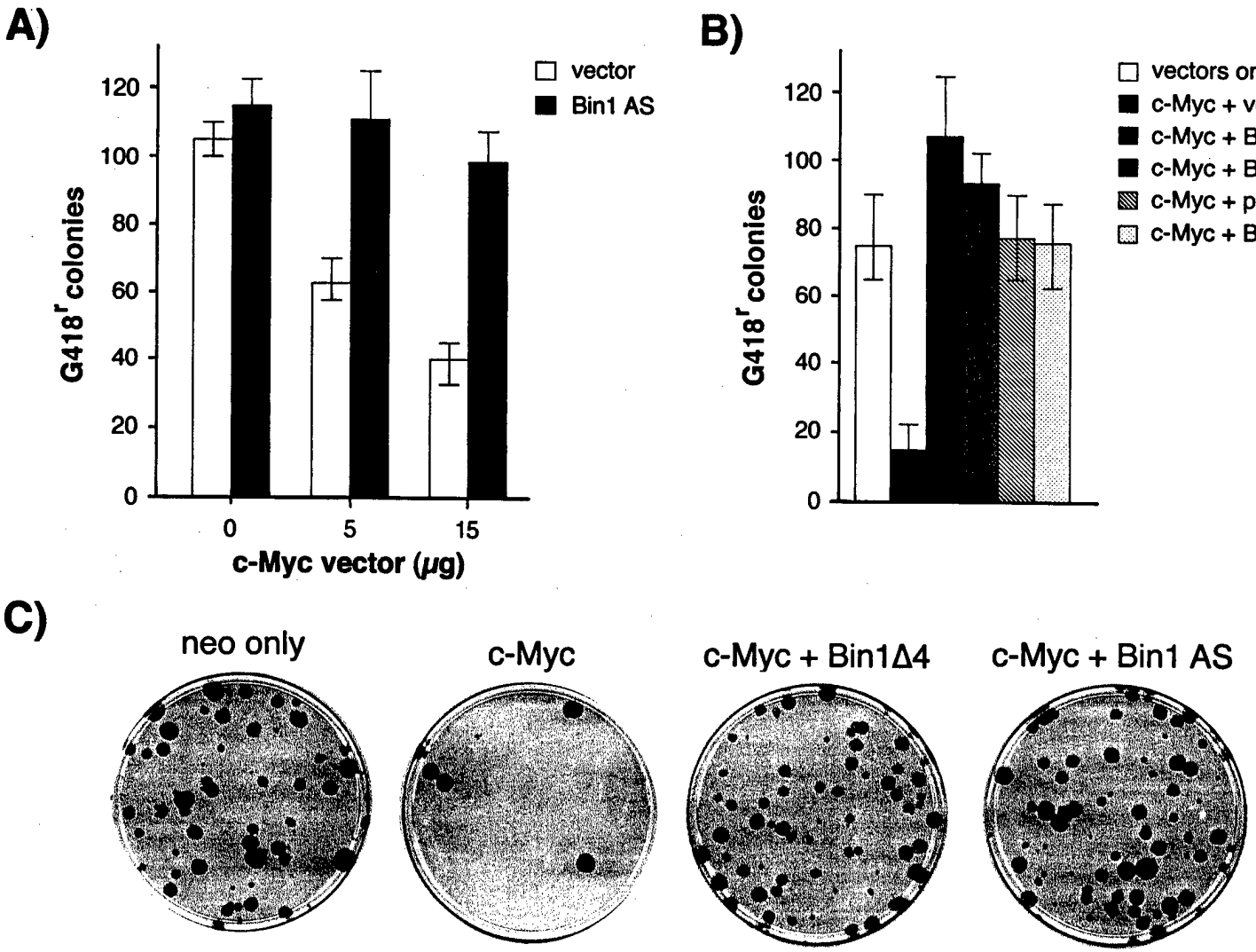
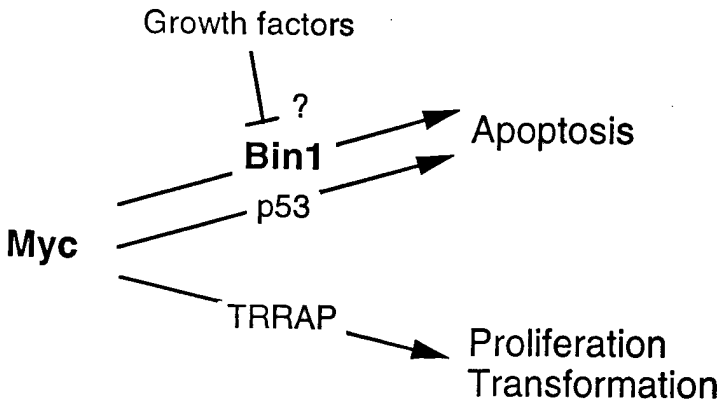

A)
G418r colonies
120
100
80
60
40
20
0
0
5
15
c-Myc vector (μg)
vector
Bin1 AS
B)
G418r colonies
120
100
80
60
40
20
0
vectors only
c-Myc + vector
c-Myc + Bin1 AS
c-Myc + Bin1Δ4
c-Myc + p53ts
c-Myc + Bcl-2
C)
neo only
c-Myc
c-Myc + Bin1Δ4
c-Myc + Bin1 AS

Fig. 6


Growth factors
?
Bin1
Apoptosis
p53
Myc
TRRAP
Proliferation
Transformation